CLIENT COPY

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

To: ROBERT W. ESMOND
STERNE, KESSLER, GOLDSTEIN & FOX P. L. L. C.
1100 NEW YORK AVENUE, N. W. -SUITE 600
WASHINGTON, D.C. 20005-3934

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

Applicant's or agent's file reference 1797.009PC05	Date of Mailing *(day/month/year)* **FOR FURTHER ACTION** **See paragraphs 1 and 4 below**
International application No. PCT/US99/26443	International filing date *(day/month/year)* 10 NOVEMBER 1999
Applicant CHANEY, RUFUS L.	

1. [X] The applicant is hereby notified that the international search report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the international search report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in rules 90 *bis* 1 and 90 *bis* 3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer MEDINA A. IBRAHIM
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

To: ROBERT W. ESMOND
STERNE, KESSLER, GOLDSTEIN & FOX P. L. L. C.
1100 NEW YORK AVENUE, N. W. -SUITE 600
WASHINGTON, D.C. 20005-3934

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

Applicant's or agent's file reference 1797.009PC05	Date of Mailing *(day/month/year)* 09 MAR 2000 **FOR FURTHER ACTION** **See paragraphs 1 and 4 below**
International application No. PCT/US99/26443	International filing date *(day/month/year)* 10 NOVEMBER 1999
Applicant CHANEY, RUFUS L.	

1. [X] The applicant is hereby notified that the international search report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the international search report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in rules 90 *bis* 1 and 90 *bis* 3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer MEDINA A. IBRAHIM
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 1797.009PC05	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
International application No. PCT/US99/26443	International filing date *(day/month/year)* 10 NOVEMBER 1999	(Earliest) Priority Date *(day/month/year)* 10 NOVEMBER 1998
Applicant CHANEY, RUFUS L.		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 5 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. [ ] **Certain claims were found unsearchable** (See Box I).

2. [X] **Unity of invention is lacking** (See Box II).

3. [ ] The international application contains disclosure of **a nucleotide and/or amino acid sequence listing** and the international search was carried out on the basis of the sequence listing
   - [ ] filed with the international application.
   - [ ] furnished by the applicant separately from the international application,
     - [ ] but not accompanied by a statement to the effect that it did not include matter going beyond the disclosure in the international application as filed.
   - [ ] transcribed by this Authority.

4. With regard to the **title**,
   - [X] the text is approved as submitted by the applicant.
   - [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,
   - [X] the text is approved as submitted by the applicant.
   - [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is:

   Figure No. _____
   - [ ] as suggested by the applicant.
   - [ ] because the applicant failed to suggest a figure.
   - [ ] because this figure better characterizes the invention.
   - [ ] None of the figures.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/26443

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/26443

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C21B 9/00; C22B 9/00; A01H 3/02, 5/00; A01G 1/00
US CL :75/710; 800/276, 260; 210/602, 681, 682
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 75/710; 800/276, 260; 210/602, 681, 682

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN CAS, DIALOG, WEST1.2a
TERMS: PHYTOEXTRACTION, PHYTOMINING, PHYTOREDUCTION, NICKEL, COBALT, HYPERACCUMULATORS, ALYSSUM, SOIL PH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X - Y	US 5,711,784 A (CHANEY et al) 27 JANUARY 1998, see whole document.	1-4, 8-14, 19-22, 29-30 ---------- 15-18, 23-40
X - Y	US 5,785,735 A (RASKIN et al.) 28 JULY 1998, see columns 6-7.	19, 41-43, ---------- 20-22,
Y	RASKIN et al. Bioconcentration of Heavy Metals by Plants. Current Opinion in Biotechnology. 1994, Vol. 5, pages 285-290, see pages 287-288.	5-7, 44-47

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 27 JANUARY 2000	Date of mailing of the international search report 09 MAR 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer MEDINA A. IBRAHIM Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/26443

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ROMERO et al. Metal Plant and Soil Pollution Indexes. Water, Air, and Soil Pollution. 1987, Vol. 34, No. 4, pages 347-352, see entire document.	1-4, 8-18, 23-40
Y	KUMAR et al. Phytoextraction: The Use of Plants to Remove Heavy Metals from Soils. Environ. Sci. Technol. 1995, Vol. 29, no. 5, pages 1232-1238, see entire document.	19-22, 41-43
Y,P	ROBINSON et al. Soil Amendments Affecting Nickel and Cobalt Uptake by Berkheya coddii: Potential Use for Phytomining and Phytoremediation. Annals of Botany. 1999, Vol. 84, pages 689-694, see page 691-694.	5-7, 44-47
Y,P	US 5,927,005 A (GARDEA-TORRESDEY et al.) 27 July 1999, see entire document.	1-4, 8-18, 23-40
A,P	US 5,928,406 A (SALT et al.) 27 July 1999, see entire document.	19-22, 41-43
Y,P	US 5,917,117 A (ENSLEY et al.) 29 June 1999, see columns 1-2, 6-8, 13-14.	5-7, 44-47
Y,P	US 5,944,872 A (CHANEY et al.) 31 August 1999, see entire document.	1-4, 8-18, 23-40

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/26443

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s)1-4, 8-18, 23-40, drawn to a method for selectively recovering nickel by elevating soil pH.
Group II, claim(s) 19-22, 41-43, drawn to a method for recovering cobalt from contaminated soil by lowering soil pH.
Group III, claim(s) 5-7 and 44-47, drawn to a method for sequentially recovering 2 metals from soil comprising the first step of raising or lowering soil pH, followed by the second step of altering pH in the opposition direction of the first step.
The inventions listed as Groups I-III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The claimed method for recovering at least one metal from metal contaminated soil by elevating the pH of soil and phytomining is anticipated by each of Chaney et al and Raskin et al, as set forth in the Search Report, and so do not constitute a single special technical feature which would be an advance over the prior art.

The invention of Group I, drawn to a first method for recovering nickel from nickel-contaminating soil, requires presence of in the soil, limestone and limestone equivalents to increase soil pH not required by any other group.

The invention of Group II, drawn to a second method for recovering cobalt from cobalt-contaminated soil, requires presence of cobalt in the soil, cobalt uptake by a plant, and a lowered pH not required by any other group.

The invention of Group III, drawn to a third method for sequentially recovering nickel and cobalt, requires a raised soil pH followed by a lowered soil pH or vice-versa not required by any other group.

CHAPTER I
PCT TELEPHONE MEMORANDUM
FOR
LACK OF UNITY OF INVENTION

PCT No.: PCT/US99/26443

Examiner: MEDINA A. IBRAHIM

Attorney spoken to: ROBERT W. ESMOND

Date of call: 21 JANUARY 2000

☒ Amount of payment approved: $420.00

☒ Deposit account number to be charged: 19-0036

☒ Attorney elected to pay for ALL additional inventions

☐ Attorney elected to pay only for the additional inventions covered by

☐ Group(s):

– encompassing –

☐ Claim(s):

☐ Attorney elected **NOT** to pay for any additional inventions, therefore, only the first claimed invention (Group I) covered by Claim(s) _ has been searched.

☒ Attorney was orally advised that there is no right to protest for any group not paid for.

☒ Attorney was orally advised that any protest must be filed no later than 15 days from the mailing of the Search Report (PCT/ISA/210).

Time Limit For Filing A Protest

Applicant is hereby given 15 days from the mailing date of this Search Report in which to file a protest of the holding of lack of unity of invention. In accordance with PCT Rule 40.2, applicant may protest the holding of lack of unity only with respect to the group(s) paid for.

Detailed Reasons For Holding Lack Of Unity Of Invention:

Detailed Reasons For Holding Lack of Unity Of Invention:
(Continued on a separate sheet)

Note: A copy of this form must be attached to the Search Report.

ATTACHMENT TO CHAPTER I PCT TELEPHONE MEMORANDUM FOR LACK OF UNITY OF INVENTION

Detailed Reasons For Holding Lack Of Unity Of Invention:
This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s)1-4, 8-18, 23-40, drawn to a method for phyto-recovering nickel from metal-contaminated soil by elevating soil pH.
Group II, claim(s) 19-22, 41-43, drawn to a method for phyto-recovering cobalt from contaminated soil by lowering soil pH.
Group III, claim(s) 5-7 and 44-47, drawn to a method for sequentially phyto-recovering 2 metals from soil comprising the first step of raising or lowering soil pH, followed by the second step of altering pH in the opposition direction of the first step.
The inventions listed as Groups I-III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The claimed method for recovering at least one metal from contaminated soil by elevating or lowering the pH of soil and phytomining is anticipated by each of Chaney et al and Raskin et al, as set forth in the Search Report, and so do not constitute a single special technical feature which would be an advance over the prior art.

The invention of Group I, drawn to a first method for recovering nickel from nickel-contaminating soil, requires presence of nickel in the soil, limestone and limestone equivalents to increase soil pH not required by any other group.

The invention of Group II, drawn to a second method for recovering cobalt from cobalt-contaminated soil, requires presence of cobalt in the soil, and a lowered pH not required by any other group.

The invention of Group III, drawn to a third method for sequentially recovering nickel and cobalt, requires a raised soil pH followed by a lowered soil pH or vice versa not required by any other group.

PATENT COOPERATION TREATY

RECD 16 OCT 1998

PCT

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 274709127CIP	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/US97/15109	International filing date *(day/month/year)* 29 AUGUST 1997	Priority date *(day/month/year)* 30 AUGUST 1996
International Patent Classification (IPC) or national classification and IPC IPC(6): C22B 23/00 and US Cl.: 75/710		
Applicant CHANEY, RUFUS L.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 3 sheets.

☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of 0 sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☐ Non-establishment of report with regard to novelty, inventive step or industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
18 FEBRUARY 1998	07 AUGUST 1998
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer M. ALEXANDRA ELVE Telephone No. (703) 308-0661

Form PCT/IPEA/409 (cover sheet) (January 1994)★

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US97/15109

I. Basis of the report

1. This report has been drawn on the basis of *(Substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments)*:

[X] the international application as originally filed.

[X] the description, pages 1-35 , as originally filed.
pages NONE , filed with the demand.
pages NONE , filed with the letter of _____.
pages _____ , filed with the letter of _____.

[X] the claims, Nos. 1-11 , as originally filed.
Nos. NONE , as amended under Article 19.
Nos. NONE , filed with the demand.
Nos. NONE , filed with the letter of _____.
Nos. _____ , filed with the letter of _____.

[X] the drawings, sheets/~~fig~~ 1-10 , as originally filed.
sheets/~~fig~~ NONE , filed with the demand.
sheets/~~fig~~ NONE , filed with the letter of _____.
sheets/~~fig~~ _____ , filed with the letter of _____.

2. The amendments have resulted in the cancellation of:

[X] the description, pages NONE.

[X] the claims, Nos. NONE.

[X] the drawings, sheets/~~fig~~ NONE.

3. [ ] This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the ~~Supplemental Box~~ Additional observations below (Rule 70.2(c)).

4. Additional observations, if necessary:

NONE

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US97/15109

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. STATEMENT

Novelty (N)	Claims	NONE	YES
	Claims	1-11	NO
Inventive Step (IS)	Claims	NONE	YES
	Claims	1-11	NO
Industrial Applicability (IA)	Claims	1-11	YES
	Claims	NONE	NO

2. CITATIONS AND EXPLANATIONS

Claims 1-11 lack novelty under PCT Article 33(2) as being anticipated by Raskin et al. (US Pat. 5,364,451). Claims 1-11 lack an inventive step under PCT Article 33(3) as being obvious over Raskin et al. Metal ions are removed from the soil by the plant family Brassicaceae which absorbs metals into their roots. Absorbed metals are then transferred to the plant shoots which are harvested (abstract). Plants accumulate metal content of approximately 30% dry weight of plant root and 3.5% dry weight of plant shoot (col. 1, lines 45-52). Metals can include Hg, Cd, Co, Ni, Mo, Cu, As, Se, Zn, Sb, Be, Au, Ba, Mn, Ag, Tl, Rb, Sr, Y, Tc, Ru, Pd, Ir, V, CS, U, Pu, Ce, Pb & Cr (col. 1, lines 52-60). Preferred plant members are Brassica species selected from the group consisting of B. juncea and B. oleracea (col. 2, lines 7-9). Claim 1-11 meet the criteria set out in PCT Article 33(4), because methods of methods of accumulating metals from soils into shoots of a plant have industrial applicability.

---------- NEW CITATIONS ----------

NONE

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :		(11) International Publication Number:	WO 98/08991
C22B 23/00	A1	(43) International Publication Date:	5 March 1998 (05.03.98)

(21) International Application Number: PCT/US97/15109

(22) International Filing Date: 29 August 1997 (29.08.97)

(30) Priority Data:

60/024,928	30 August 1996 (30.08.96)	US
60/030,462	6 November 1996 (06.11.96)	US

(71)(72) Applicants and Inventors: CHANEY, Rufus, L. [US/US]; United States Department of Agriculture, Beltsville, MD 20705 (US). ANGLE, Jay, Scott [US/US]; 10241 Bristol Channel, Ellicot City, MA 21042 (US).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: LI, Yin-Ming [CN/US]; 12019 Coldstream Drive, Potomac, MD 20854 (US).

(74) Agents: KELBER, Steven, B. et al.; Oblon, Spivak, McClelland, Maier & Neustadt, P.C., Crystal Square Five, 4th floor, 1755 Jefferson Davis Highway, Arlington, VA 22202 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published

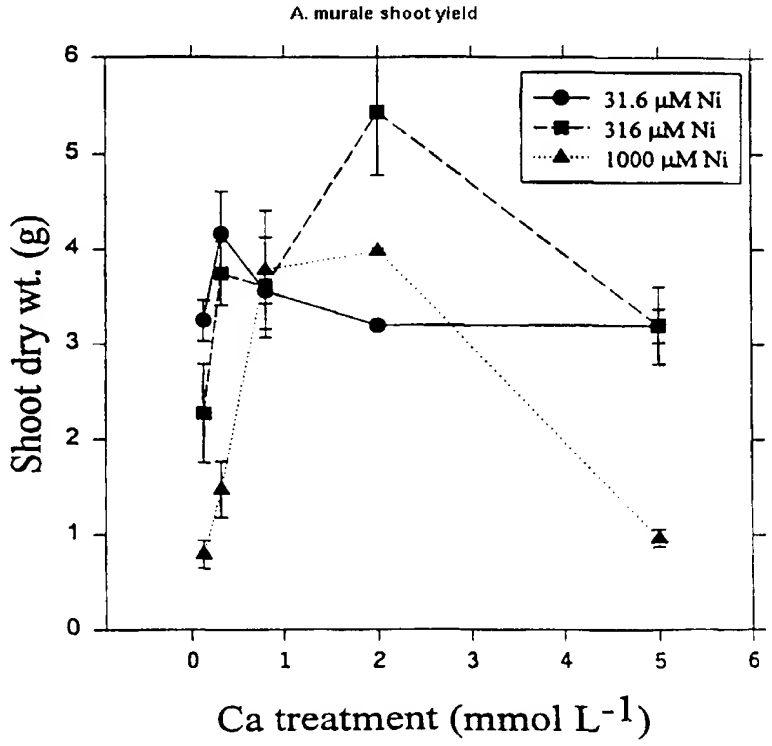
With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: METHOD FOR PHYTOMINING OF NICKEL, COBALT AND OTHER METALS FROM SOIL

(57) Abstract

The recovery of nickel, cobalt and other metals by phytomining is described. Plants of the *Alyssum* genus are grown in nickel rich soil. The uptake of nickel is enhanced by maintaining specific soil conditions, including a concentration of calcium between (but not including) 0.128 mM and 5.0 mM and an acidic pH. Ni uptake may be further enhanced by maintaining a ratio of exchangeable Ca/Mg of 0.16-0.40. Uptake may be further enhanced by addition of chelating agents and ammonium based fertilizers.



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

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CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

TITLE OF THE INVENTION

METHOD FOR PHYTOMINING OF NICKEL, COBALT AND OTHER METALS FROM SOIL

This application claims priority of U.S. Provisional Patent Application 60/024,928, filed August 30, 1996 and U.S. Provisional Patent Application 60/030,462, filed November 6, 1996.

The United States Government may have rights in this application, and the invention disclosed and claimed herein, by reason of Agricultural Research Contract No. 58-3k95-5-352.

BACKGROUND OF THE INVENTION:

Field of the Invention

This invention pertains to a method of extracting nickel, cobalt and other metals, including the platinum and palladium metal families, from soil by cultivation of the soil with hyperaccumulating plants that concentrate these metals in above-ground portions of the plants, which can be harvested, dried and smelted to recover the metal (metal phytomining).

BACKGROUND OF THE PRIOR ART

It has long been known that certain types of soil and geological materials, including serpentine, lateritic serpentine, ultramafic and meteor-impacted soils may be rich in nickel or cobalt, and are sites for mining of these metals. The cost of conventional mining for these metals remains high, and the level of metals required in geological materials to which current technology may be usefully applied are

much higher than most serpentine, lateritic serpentine, ultramafic and meteor-derived soils.

This application is related to U.S. Patent Application Serial No. 08/470,440, allowed, and its corresponding PCT application. In this earlier application, recovery of Ni, Co and related metals from soil is described through culturing *Allysum* plants on Ni-enriched soil. The specific soil conditions described in that application include reducing calcium as far as possible, in accordance with conventional teachings regarding the inverse relationship between calcium concentration and nickel hyperaccumulation. Additionally, the application limits calcium concentrations to a value such that the exchangeable Ca/Mg ratio is below 0.20.

U.S. Patent 5,364,451, Raskin et al., is directed to a method of removing metals from metal-rich soil by growing genetically altered plants of the family *Brassicaceae* in these soils, so as to remediate polluted soils at a reduced cost. Suitable parents for the mutants that are the subject of the Raskin patent include *B. juncea*. While the patent generally describes a large number of metals that may be recovered, specific artificial examples are directed to recovery of chromium and lead. The entire disclosure of U.S. Patent 5,364,451 is incorporated herein by reference.

A review of the examples of this reference, and application of the technology proposed, illustrates continuing problems posed in rededication of metal-rich soil, and recovery of the metals therefrom. In particular, the examples set forth reflect artificial culture in sand media with intermittent feeding with phosphate to permit plants to grow without severe yield reduction and without severe lead toxicity. The patent also relies on genetic mutations that are produced by random "mutagenesis", that is, the creation of a library of mutants or potential mutants from a starting parent by indiscriminate application of a mutagen, coupled with screening the offspring to define acceptable hyperaccumulators. While promising, the Raskin patent offers little basis for an opportunity to proceed directly with soil rededication

through plant growth or culturing. Additionally, the patent offers little realistic opportunity for recovery of the metal itself, indicating only that under circumstances (not identified) the metal can actually be reclaimed.

One of the most widely found, and technologically important metals is nickel. Nickel is a natural constituent in all soils, being particularly high in concentration in serpentine, lateritic serpentine, ultramafic and meteor-derived soils. Cobalt, which has chemical and geological characteristics very similar to nickel, can similarly be found in these soils, and is another valuable metal. Other metals that are also subjects for phytomining within the scope of the invention, including those of the platinum and palladium families, including palladium, rhodium, ruthenium, platinum, iridium, osmium and rhenium which commonly co-occur with Ni and Co. Cultivation of plants which are hyperaccumulators of these metals, in metal-rich soils, or "phytomining", is a desirable alternative as a means for recovering metals from soil. Ordinary cultivation methods, however, without adequate preparation and maintenance of soil conditions, does not lead to adequate hyperaccumulation of metals in the plants economically interesting. Additionally, specific methods for recovery of the metals remain to be explored.

Among the soil conditions and cultivation methods most frequently investigated, the relationship between calcium levels and nickel uptake, as well as nickel tolerance, have been frequently reported. While the reports are not uniform, in general, the prior art has reported a negative correlation between calcium concentration and nickel upgrowth. Gabbrielli et al., Atti. Soc. Tosc. Sci. Nat. B38:143-153 (1981) observed that serpentine soils typically have low levels of calcium. An increase in calcium level was reported to reduce nickel uptake. Similarly, increasing Mg and Ca has been reported to lower nickel tissue concentration in nickel accumulator species endemic to serpentine soils. Gabgrielli et al., Physiol. Plant. 62:540-544 (1984). See also, Vergnano et al., The Vegetation of Ultramafic Soil, page 319-322, (1992). Thus, in general, the art teaches that raising calcium levels from the extremely low values normally

encountered in serpentine soil to higher levels can be expected to yield a reduction in nickel uptake.

Similarly, a ratio recognized as important in maintaining the health of various plants endemic to serpentine soils is the exchangeable Ca/Mg ratio. Prior art reports set a ratio of about 0.67 recommended as a fertility index. Alexander et al., Soil Sci. 149:138-143 (1990). Typically, exchangeable Ca/Mg ratios in serpentine soils are at much lower values of about 0.2. Thus, the general teaching of the art is that to preserve fertility, a substantial increase in available calcium is required, which can be expected to decrease nickel uptake.

In U.S. Patent Application Serial No. 08/470,440, which is incorporated in its entirely herein by reference, a method of phytomining is disclosed which calls for reduction of calcium levels, among other soil treatments. This is consistent with teachings of the prior art. Accordingly, it remains an object of those of skill in the art to develop a reliable system for phytomining of soils rich in nickel, cobalt and the other identified metals, naturally occurring or otherwise, that will lead to a recovery of these metals at economically acceptable levels.

SUMMARY OF THE INVENTION

By screening a wide variety of plants from the *Brassicaceae* family, the inventors have identified plants in the *Alyssum* genus which may be hyperaccumulators of nickel and which accumulate valuable amounts of cobalt. By definition, hyperaccumulator plants accumulate over 1000 mg Ni or Co/kg dry weight growing in the soils where they evolved. Because cobalt occurs at about 3-10% of the level of Ni in the listed soils, Ni is the dominant toxic metal which induced evolutionary selection of the Ni hyperaccumulator plants and Co is accumulated to economically useful levels but Ni hyperaccumulation is the dominant economic benefit of the phytomining technology. Evidence suggests members of the section Odontarrhena of the genus *Alyssum* are likely candidates as nickel

hyperaccumulators. The plant may also concentrate, in the above-ground plant tissues, metal from the platinum and palladium families, including Pd, Rh, Ru, Pt, Ir, Os and Re, in significant amounts. Accumulation of nickel in plant tissues in excess of 2.5 percent is practicable.

The metals listed accumulate in biomass by growing nickel hyperaccumulating *Alyssum* species in the target soils. Some 48 taxa within the section Odontarrhena of the genus *Alyssum* are known to be hyperaccumulators of nickel. These include the following species already evaluated: A. murale, and A. pintodasilvae (A. serpyllifolium ssp.), A. malacitanum, A. lesbiacum, and A. fallacinum. Other Ni-hyperaccumulating species which may be employed include: A. argenteum, A. bertolonii, A. tenium, A. heldreichii. About 250 other plant taxa have been shown to hyperaccumulate nickel, but many of these do not exceed 10,000 mg Ni/kg d.w., and the majority are of tropical origin.

The identified metal species are accumulated by growing the *Alyssum* in nickel-rich soil, under specific soil conditions. The conditions include: (1) lowering the soil pH, which increases the phytoavailability of nickel; (2) maintaining moderate levels of Ca in the soil by appropriate treatments and by use of Ca, Mg-rich soil amendments adjusted to maintain Ca levels at levels corresponding to solution values between 0.128 mM and 5.0 mM; (3) using ammonium constraining or ammonium-generating nitrogen fertilizers to improve plant growth and to increase Ni hyperaccumulation due to rhizosphere acidification; and (4) applying chelating agents to the soil to improve nickel uptake by the roots of the hyperaccumulating *Alyssum* species. Examples of suitable chelating agents include nitrilotriacetic acid (NTA). Other chelating agents commonly used in connection with increasing soil metal mobility for plant uptake include ethylenediaminetetraacetic acid, and ethylene glycol-bis-(β-aminoethylethehr)-N, N-tetraacetic acid. Maintenance of these soil-conditioning factors will improve nickel hyperaccumulation in *Alyssum*, in excess of a 2.5 percent concentration in above-ground portions of the plant, particularly leaves and stems or shoots, which make

for easy cultivation and metal recovery. This is preferable to concentration in the roots, discussed in Raskin et al., which may be an aid in soil rededication if non-leachable therefrom, but does not offer convenience for phytomining. It is particularly surprising that intermediate values of Ca increase Ni uptake while values of 0.128 mM and below and 5 mM and above decrease Ni uptake. This, combined with exchangeable Ca/Mg ratios of 0.16-0.40, much lower than that recommended in the prior art, further increases Ni tissue concentrations.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1 -10 are graph illustrations of experimental data obtained and discussed below.

Figures 1-3 reflect shoot yield for given levels of Ni as a function of Ca concentration for Cabbage, *A.murale* and *A. pintodasilvae,* respectively.

Figures 4-6 reflect Ni levels in shoots for given levels of Ni as a function of Ca concentration for Cabbage, *A.murale* and *A. pintodasilvae,* respectively.

Figures 7-8 reflect the ratio of Ni in shoots/roots for *A.murale* and *A. pintodasilvae,* respectively.

Figures 9-10 reflect shoot Ni content at five given Ni concentration values as a function of Ca concentration for *A.murale* and *A. pintodasilvae,* respectively.

DETAILED DESCRIPTION OF THE INVENTION

Applicants have screened a large wild-type collection of germplasm to identify hyperaccumulating plants. In particular, plants of the Brassicaceae family, particularly naturally occurring plants as opposed to those with induced mutations, such as those employed in the Raskin patent, are known to be Ni + Co accumulators. Within the family, and even with the various genera, however, wide variations in metal accumulation, to the extent it occurs, do appear. *Alyssum*

species that are preferred candidates for use in the claimed invention concentrate and hyperaccumulate nickel, shown enhanced uptake of cobalt and may be useful in accumulating other metals. Preferred species have a preference for, and a high toxicity resistance to these metallic elements. This appears to be due to evolutionary driving forces, which permit the plant to benefit from the ecological niche presented. This should be contrasted with the response of a different Brassicaceae member, Thlaspi caerulescens, which accumulates very high levels of zinc and cadmium. While *Alyssum* exhibits a higher uptake rate at low nickel and cobalt concentrations than other species, Thlaspi actually grow well on soils with much higher Zn and Cd concentrations. Thus, while *Alyssum* concentrates nickel and cobalt over a range of concentrations, Thlaspi hyperaccumulates very high levels of Zn and Cd, some strains accumulating Ni and Co. Rather than relying on the unpredictable process of mutagenesis, the applicants in screening a large library of wild-type germplasm, have identified several *Alyssum* species including A. murale, A. pintodasilvae (A. serpyllifolium ssp.), A. malacitanum, A. lesbiacum, A. tenium and A. fallacinum as a suitable hyperaccumulators of nickel land useful in the enhanced uptake of cobalt. The same plants may also accumualte Pd, Rh, Ru, Pt, Ir, Os and Re. While these platinum and palladium metals are accumulated in lower concentrations, their greater value per unit weight, makes phytomining of these metals economically attractive as well.

Soil Management

To improve nickel and cobalt sequestration in the above-ground tissues of *Alyssum* plants, the soil in which they are grown is preferentially conditioned taking advantage of different factors.

These include soil pH, moderate calcium concentrations low to moderate exchangeable Ca/Mg ratios, and optionally, use of ammonium containing or generating fertilizer rather than other N-fertilizers and application of chelating agents. Each of these is considered in turn below.

Soil pH

The maintenance of preferred pH ranges in soil is well known in agriculture for a variety of reasons. Typically, pH of soil is altered or modified so as to maintain it within a near neutral range of about 6.0-7.5. Thus, soil near a limestone foundation or other building may be treated with acidifying soil amendments so as to reduce an alkaline pH. Soil with a naturally low pH may instead be treated with limestone or similar amendment, so as to increase the soil pH. A reduced pH increases the phytoavailability of nickel and cobalt. A reduced pH increases solubility and optimizes the release of these metals for absorption by the roots, and translocation to the above-ground tissues of the plant. Soil pH can be maintained in any of a variety of established methods, and the methods themselves do not constitute an aspect of this invention. Preferably, soil pH is managed at a low value by addition of sulfur and use of ammonium - N fertilizers. The *Alyssum* species, and indeed, any plant species, grows best at its evolved optimum pH conditions. Thus, pH cannot be reduced so low as to substantially retard or inhibit plant growth. An optimum pH range for phytomining using *Alyssum* is a pH of 4.5 to 6.2, preferably 5.2-6.2. After extraction of economically phytominable Ni and Co from the soil, limestone application can raise soil to pH levels required by more traditional farm crops.

Calcium Concentrations

Alyssum species which hyperaccumulate Ni and Co evolved in Ni-rich ultramafic and serpentine soils which simultaneously have low soil calcium. The presence of extremely low and extremely high calcium concentrations in soil inhibits nickel/cobalt hyperaccumulation by *Alyssum*. Acceptable calcium concentrations in soil ranges from 0.128 mM to 5.0 mM, as set forth in the examples below. Calcium concentrations may be maintained by any of a variety of known methods. One method involves acidification of the soil with sulfur, sulfuric acid, or other amendments and leaching, followed by use of Ca soil amendments.

Whatever method is selected to adjust calcium concentration in soil, it should be selected so as to be consistent with the objective of soil phytomining.

Additional of Ammonium Fertilizer

Generally, high metal concentrations are toxic to plants, and inhibitory of plant growth. While *Alyssum* has developed the ability to hyperaccumulate nickel/cobalt in its above-ground plant tissues, nonetheless, fertilizer support for the growth, particularly in polluted soil, is an essential element for substantial hyperaccumulation. Use of high-ammonium N-fertilizers is of value. Nonetheless, the use of ammonium fertilizers per se is well known, and acceptable fertilizers and protocols will be arrived at by those of ordinary skill in the art on an empirical basis.

Addition of Chelating Agents

Metal chelates are commonly used in agriculture, and occur naturally is living cells. The addition of chelating agents, such a NTA, or any of a variety of amino-acetic acids known to those of ordinary skill in the art as chelating agents, to the soil to be phytomined for Ni/Co and Pt, Pd metals improves the movement of soil metals to root surfaces for uptake and translocation of these materials into the above-ground plant tissues. Any of a variety of known chelating agents of commerce may be used. A preferred chelating agent is NTA or EDTA. Typically, chelating agents will be added at 5-100 kg/ha after the plants are established. As with the use of fertilizers, optimum additions of chelating agents can be determined on an empirical basis. Chelating compounds which chelate Ni int eh presence of high soil levels of Fe, Mg, and Ca selectively increase Ni uptake by the hyperaccumulator plants.

Metal Recovery

As noted, a principal object of this invention is the recovery of the metal sequestered by the hyperaccumulating plant. In U.S. Patent 5,364,451, plants are identified which accumulate the metals in the roots. Recovery of metals from roots poses substantial mechanical problems, including the recovery of the root itself, as well as recovery of the metal from the root tissue. By cultivating selected *Alyssum* genotypes, as contemplated in the claimed invention, a very high degree of the nickel/cobalt absorbed by the roots is translocated to above-ground tissues, such as stems, leaves, flowers and other leaf and stem tissues. This feature facilitates recovery of the metal extracted from the soil. The *Alyssum* can be harvested in conventional fashion, that is, cutting of the plant at soil level. The harvested materials are left to dry, in much the same fashion that alfalfa is dried, so as to remove most of the water present in the plant tissues. After drying, the plant material is collected from the field by normal agricultural practices of hay-making, incinerated and reduced to an ash with or without energy recovery. This organic material may alternatively be further treated by roasting, sintering, or smelting methods which allow the metals in an ash or ore to be recovered according to conventional metal refining methods such as acid dissolution and electrowinning. With metal concentrations as high as 2.5 to 5.0% in the above-ground plant tissues, particularly leaves or shoots, metal recovery becomes economical, thus satisfying the primary objective of the invention. Conventional smelting/roasting/sintering temperatures of 500-1500°F are sufficient to combust the organic material in the dried plant biomass, leaving a residue of the accumulated metal, with few contaminants which are known to interfere with metal refining. Indeed, it is suspected that the other components of the ash will be lower than with conventional mined ore concentrates.

EXAMPLES

Materials and Methods

Plant Material

A nutrient solution study was conducted to define the effects of Ca and Mg on Ni uptake by two know Ni hyperaccumulator species, *Alyssum murale* and *Alyssum pintodasilvae*, compared to the normal non-tolerant crop species, cabbage (Brassia oleracea var. *capitata)* cultivar Danish Roundhead. A varying solution concentrations of Ni (3 levels) and Ca (5 levels) were used in a factorial experimental design for *Alyssum,* while 2 levels of Ni and 5 levels of Ca were used in a factorial experimental design for cabbage. All solutions contained a high concentration of Mg to simulate serpentine soils where phytomining plants might be grown. Seeds for *Alyssum murale* and *Alyssum pintodasilvae* were collected from plants growing in Panorama, Thessaloniki, N. Greece and Braganca, NE Portugal.

Plant Growth

The study was conducted in an environmental growth chamber; temperature in the chamber was maintained at 25°C day and 19°C night, and relative humidity was set at 70%. The day period was maintained for 16 hours periods with >400 $\mu Em^{-2}sec^{-1}$ photosynthetically active radiation at plant height from a combination of cool-white fluorescent and incandescent lamps.

Alyssum seeds were treated with ethanolic Arasan for 45 seconds and germinated by placing seeds in company germination bags with a macronutrient solution (1 mM Mg as $MgSO_4$; 2.5 mM $CaNO_3$ and KNO_3; 0.1 mM K_2HPO_4). The bags were kept moist all the time. After 2 weeks in the germination bags in the growth chamber, *Alyssum* seedlings were transferred into 8 L buckets containing a 0.5 strength Hoagland solution (1 mM Mg as $MgSO_4$; 2.5 mM $CaNO_3$ and KNO_3; 0.1 mM K_2HPO_4; 20 μM Fe as FeHBED; 75 μM KCl; 25 μM HCl; 10μM H_3BO_3; 2 μM Mn as $MnCl_2$; 05 μM Cu as $CuSO_4$; and 0.2 μM Mo as Na_2MoO_4; 1.0 mM

Zn as $ZnSO_4$). Seedlings were maintained in these buckets for an additional 2 weeks to grow to larger or reasonable handling before transplanting to treatment solutions.

Cabbage seeds germination was begun 10 days before transplanting to treatment solutions. Cabbage seeds were placed in standard seed germination papers with the same germination macronutrient solution and showed good germination within six days.

To initiate treatments, one plant of each species was transferred to separate 1 L polyethylene beakers containing a modified 0.5 strength Hoagland solution (2 mM MG as $MgSO_4$; 2.5 mM KNO_3; 0.1 mM K_2HPO_4; 20 μM Fe as FeHBED; 75 μM KCl; 25 μM HCl; 15 μM H_3BO_3; 2 μM Mn as $MnCl_2$; 0.5 μM Cu as $CuSO_4$; 0.2 μM MO as $NaMoO_4$; and 1.0 mM Zn as $ZnSO_4$) with 2 mM MES to maintain solution pH at 6.2, high Mg level (2 mM) and Ca and Ni treatments. FeHBED was used because even high levels of Ni or micronutrients do not displace Fe from this chelate, and dicots easily obtain the Fe by reduction.

A randomized complete block design with three replications was used. The plants were placed into polyurethane foam plant supports and inserted into a slot and hole in a black plexiglass cover. The beakers were covered with black polyethylene to minimize light exposure. Each beaker was continuously aerated.

Plants were harvested six weeks after treatment initiation. At harvest, plants were separated into roots and shoots. Shoots were rinsed with deionized water. Roots were rinsed with 2.5 mM $Ca(NO_3)_2$ to remove extracellular metals prior to rinsing with deionized water. All samples were dried at 65°C in a forced draft oven.

Treatments

Ni was supplied as $NiSO_46H_2O$. Three high concentrations Ni treatments were established for the *Alyssum* spp. (31.6 μM, 316 μM, and 1000 μM), and two Ni treatments were established for cabbage (1.0 μM and 10.0 μM) based on

preliminary studies of Ni tolerance by these species. Ca was supplied as $Ca(NO_3)_2 4H_2O$ with NH_4NO_3 to adjust nitrogen concentration to 10 mM for all treatments. Five Ca treatments were established for all tested species 0.128 mM, 0.32 mM, 0.8 mM, 2.0 mM, 5.0 mM $Ca(NO_3)_2$ with balancing 4.87 mM, 4.68 m<, 4.2 mM, 3.0 mM, 0.0 mM NH_4NO_3. Solution pH was maintained above 6.0 by the addition of 2 mM MES buffer. pH was adjusted as necessary by the addition of KOH. Fourteen days after initiation of treatment, all solutions were completely replaced, and again at 21, 28, 28 and 35 days of treatment.

Sample Analysis

Dry plant samples were ground in a stainless steel Wiley mill if necessary, 2.00 g samples were weighed into low silicate beakers and ashed in a 480°C muffle oven for 16 hours. Ash was digested with 2 mL concentrated HNO_3 and heated to incipient dryness; 10 mL 3N HCl was added, the beakers heated at reflux with stirring for 2 hours. Digests were filtered, a 1.00 mL aliquot of Fisher Scientific Cobalt Reference Solution (1000 mg L^{-1} Co) was added to each sample as an internal reference (40 mg L^{-1} cobalt) for subsequent analysis using Inductively Coupled Plasma Emission Spectrophotometry (ICP-ES). Samples were brought to 25 mL in 1N HCl. Necessary dilutions were made in 1N HCl to maintain constant viscosity. Blanks were prepared for every 10 samples and NBS#1575 pine needles standard reference materials were digested for every 20 samples for quality assurance. Plant analysis was performed in duplicate when there was sufficient sample. Ni concentration of plants were determined using a flame atomic absorption spectrometer (AA). Zn, P, Cu, Mn, Fe, Mg, Ca, and K concentrations were analyzed by using an ICP-ES (emission spectrometer), and all results were corrected by use of the internal standard.

Statistical Analysis

Data was analyzed using SAS-PC version 6.0 (SAS institute, 1989). Data required lot transformation to attain homogeneity. The GLM procedure was utilized for analysis of variance of plant yield and tissue metal concentration for differences of treatments. Treatment means were compared using the Duncan K-ratio t-test after it was determined that there was a significant ($P < 0.05$) treatment effect using the GLM procedure.

Growth and Symptoms

The experiment tested for interactions between Ca and Ni in growth and element accumulation in cabbage and two Ni hyperaccumulator *Alyssum* species. *Alyssum murale, A. pintodasilvae*, and cabbage plants all appeared healthy at the start of Ni and Ca treatments.

In the first week of growth, *Alyssum* spp. and cabbage plants in al treatments were green. In the second week of the trial, those *Alyssum* spp. plants in highest solution Ni level (100 μM) with lowest solution Ca (0.128 mM) and highest solution Ca (5 mM) started to show chlorotic symptom on young leaves, but the size was not significantly different; and those cabbage in higher solution Ni level (10 μM) almost all showed chlorotic or blown spots symptoms with curling edge in young leaves. At the fourth week of the trial, those *Alyssum* spp. plants in highest solution Ni level (1000 μM) with lowest solution Ca (0.128 mM) and highest solution Ca (5 mM) were visibly smaller than others; the chlorotic and necrotic symptoms in those cabbage in higher solution Ni level became more severe.

In the sixth week of the trial and just before harvesting, little chlorotic leaves symptoms were observed on those *Alyssum murale* plants in lowest solution Ca level (0.128 mM) across all three solution Ni levels. Smaller size and severe chlorotic symptom were significantly showed on those *Alyssum* plants in highest solution Ni level with lowest solution Ca level. For cabbage plants, not just those plants grown in higher solution Ni level showed chlorotic and necrotic with curling

edge symptoms, but also showed on the lower solution Ca levels (0.128, 0.32 mM) in lower solution Ni. Root systems were less extensive in all plants shown severe chlorotic and/or necrotic symptoms in leaves.

For all species, low Ca (0.128-32 mM) caused reduced yield compared to normal (0.8-2.0 mM) or high solution Ca (5.0 mM), for all Ni levels. Cabbage was more sensitive to Ni phytotoxicity, than the *Alyssum* species and low Ca caused greater toxicity than in *Alyssum*. For cabbage (Fig. 1), at 1.0 μM Ni, full yield was restored by increasing solution Ca; but at 10 μM Ni, full yield was not restored at higher Ca levels. But for *Alyssum* species (Fig. 2 and 3), yield also declined at 5 mM Ca.

In tables 1A - 1D, the analysis of variance for the main factors (solution Ca, solution Ni, plant species, block) and interactions (solution Ca-x-solution Ni within species, and solution Ca-x-solution Ni-x-species) are reported. All the main factors and interactions, except block, had significant effects ($P < 0.001$) on shoot dry yield and shoot Ni concentration.

Dry Matter Yields

Cabbage

For the lower solution Ni level (1 μM), increasing solution Ca had hyperbolic effect with decreasing slope in increasing shoot dry yield (Fig. 1). For the higher solution Ni (10 μM), increasing Ca caused a 5 times shoot yield increase at 2 mM Ca, but declined at 5 mM Ca when compared to 2 mM Ca levels. The similar pattern was observed on root dry yield.

Alyssum murale

For the lower solution Ni level (31.6 μM), increasing solution Ca increased shoot dry yield (Fig. 2) up to 0.32 mM Ca and caused a progressive decline after that. For the higher solution Ni levels (316 and 1000 μM), increasing solution Ca caused shoot yield to increase 2.5-4 fold up to 2 mM Ca, but declined at 5 mM Ca.

The similar pattern was observed on root dry yield in lower Ca levels, but the higher Ca levels only caused a small decline in root yield and the difference was not significant.

Alyssum pintodasilvae

For the lower solution Ni levels (31.6 and 316 μM), increasing solution Ca increased shoot dry yield (Fig. 3) up to 2 times up to 0.8 mM Ca and decreased yield at 5 mM Ca. A similar pattern was observed for the highest solution Ni level (1000 μM), but the highest shoot yield was obtained at 2 mM. For the lower solution Ni level (31.6 μM), increasing solution Ca had hyperbolic effect with decreasing slope on root yield (Fig. 3A1). For the middle solution Ni level (316 μM), increasing solution Ca increased shoot yield up to 0.8 mM Ca, with a progressive decline at higher Ca. For the high solution Ni level (1000 μM), a trough effect with positive slope in lower Ca levels was observed.

Across all Ni and Ca treatments (Table 3), dry matter yield of shoot, root, and whole plant were found significantly different ($P < 0.0001$) for three species tested, except the root yield of *Alyssum murale* which was only significantly difference in $P < 0.05$ level. the maximum shoot and root yield were attained at 31.6 μM Ni with 2 mM Ca for *Alyssum murale*, at 31.6 μM Ni with 0.8 Ca for *Alyssum murale*, and at 1.0 μM Ni with 2 mM for cabbage.

Ni Concentration and Distribution in Dry Matter

Cabbage

For the lower solution Ni level (1.0 μM), increasing solution Ca had no effect on shoot Ni concentration (Fig. 4). For the higher solution Ni (10 μM), increasing Ca caused a progressive decline in shoot Ni up to 2 mM Ca but did not decrease further at 5 mM Ca. The similar pattern was observed on root Ni concentration for the lower solution Ni level. A trough effect with positive slope at higher Ca levels was observed at higher solution Ni level (10 μM).

Alyssum murale and *A. pintodasilvae*

For the lower solution Ni level, increasing solution Ca decreased shoot Ni (Figs. 5, 6) somewhat, with flat response after 0.8 mM Ca. But for the higher solution Ni levels, increasing solution Ca decreased shoot Ni at low Ca, but increased shoot Ni at high solution Ca. For *Alyssum pintodasilvae,* Ni was so toxic at the lowest Ca with 1000 μM Ni that the reduction in shoot Ni with increasing Ca at low Ca levels (0.128 to 0.32 mM) was not observed, in contrast with the pattern for 31.6 and 316 μM Ni.

For the lower solution Ni level, increasing solution Ca had a flat response on root Ni concentration of *Alyssum murale*, but decreased root Ni somewhat with flat response after 0.32 mM Ca for *A. pintodasilvae*. For the higher solution Ni levels (316 and 1000 μM), increasing Ca decreased root Ni in low Ca levels and increased root Ni after 2 mM Ca, but increasing solution Ca had no effect on root Ni at low Ca with 316 μM solution Ni for *A. murale*.

Alyssum species translocated a greater percentage of Ni to shoot tissue. Shoot contained was 84% to 98% of total plant Ni acrose all Ni and Ca treatments. Shoot Ni/root Ni concentration ratio values ranged from 1 to 10 (Figs. 7,8), far higher than found in cabbage or in tomato (Chaney et al. 1997).

Across Ni and Ca treatments, dry matter yield, Ni concentration, and Ca concentration differences of shoot, root, and whole plant were found for the three species tested ($P < 0.001$), except root yield of *Alyssum murale* was only significantly difference in $P < 0.05$ and root Ca concentration of *A. murale* had no significantly difference (Table 3).

Nutrient Composition in Shoot Dry Matter

Zn concentration

Shoot Zn concentration in *Alyssum* spp. (Table 5A and 5B) were significantly higher in the highest solution Ca levels in 1000 μM Ni treatment, and remained similar across all Ca treatments for lower solution Ni levels. The highest

shoot Zn concentration in *Alyssum* spp. Was observed in highest solution Ca with highest solution Ni level. However, shoot Zn concentration in cabbage (Table 5C) was significantly lower in the higher solution Ca levels for both solution Ni levels, and the highest shoot Zn concentration in cabbage was found at the highest solution Ni level with lower solution Ca levels. In general, these interaction did not cause plant Zn to be raised to toxic levels or reduced to deficient levels. In crop plants, Ni is commonly found to reduce shoot Zn concentration and had additive effect to each other when concentration is above their toxic threshold (Wallace and Berry, 1989).

Cu and Mn concentration

Shoot Cu and Mn concentration in *Alyssum* spp. (Table 5A and 5B) were highest in the highest solution Ca treatment for higher solution Ni levels (316 and 1000 μM) and remained similar across all Ca treatments for lower solution Ni level (31.6 μM). The highest shoot Cu concentration in *Alyssum* spp. was observed in highest solution Ca with highest solution Ni level. Shoot Cu and Mn concentration in cabbage (Table 5C) decreased with increasing solution Ca for all solution Ni, except that there was no significant difference for Cu uptake in lower solution Ni level.

Fe concentration

For *Alyssum murale* (Table 5A), shoot Fe concentration was lowest in the highest solution Ca treatment in lower solution Ni (31.6 μM), and remained similar across all Ca treatments in highest solution Ni (1000 μM). For all solution Ni levels in *Alyssum pintodasilvae* (Table 5B) and middle solution Ni level (316 μM) in *A. murale*, shoot Fe concentration was highest in the normal solution Ca levels (0.8-2 mM) and lower in both lower and higher Ca treatments. The highest shoot Fe concentration in cabbage (Table 5C) was happened in lowest solution Ca with lower solution Ni level.

P concentration

Shoot P concentration in *Alyssum* spp. (Table 5A and 5B) in higher solution Ni levels was lower in the normal solution Ca levels and higher in both higher and lower solution Ca levels, and remained not significantly different in lower solution Ni level. Increasing solution Ca level decreased shoot P concentration in cabbage (Table 5C) across both solution Ni levels. Shoot P was in the normal range for healthy plant growth in all treatments, the adequate shoot P concentration is 2 g/kg for most plants (Taiz and Zeiger, 1991).

Mg and Ca concentration

Shoot Mg concentration decreased with increasing solution Ca level across all Ni treatments and species (Table 5A, 5B, and 5C), and shoot Ca concentration increased regularly with increasing solution Ca.

Correlation between Ni and All Other Elements Concentration in Shoot Dry Matter

There was no significant correlation between Ni concentration and Mg and Ca concentration in shoot for all species (Table 4A, 4B, and 4C), except *Alyssum* spp. had a positive correlation between Ni concentration and Ca concentration ($P < 0.05$).

A positive correlation ($P < 0.001$) between Ni concentration and Zn, Cu, and P concentration in shoot for all species was observed. The correlation between shoot yield and shoot Ni concentration was negative ($P < 0.001$ for *Alyssum murale* and cabbage, but only $P < 0.01$ for *Alyssum pintodasilvae*).

Only *Alyssum pintodasilvae* has negative correlation ($P < 0.001$) between Ni concentration and M n and Fe concentration in shoot. Cabbage had positive correlation ($P < 0.001$) between Ni concentration and Mn concentration in shoot.

Ni Content

Shoot Ni content showed a similar pattern to shoot Ni concentration in *Alyssum murale* (Fig. 9), but the shoot yield was reduced remarkably due to toxicity of 1000 μM Ni combined with high Ca level (5 mM) and caused the reduction of shoot Ni content. Shoot Ni content of *Alyssum pintodasilvae* (Fig. 10) reflected the pattern of shoot yield, except the lowest solution Ni level was no difference due to low Ni concentration in shoot.

The best treatment to get maximum Ni content in shoots was 316 μM Ni with 5 mM Ca for *Alyssum murale* (50 mg/plant) and 1000 μM Ni with 2 mM Ca for *Alyssum pintodasilvae* (40 mg/plant) in 6 weeks growth period. Cabbage shoots contained only less than 1.5 mg Ni/plant in all conditions.

Table 1A. Mean squares (MS) for the combined analyses over species, Ni treatments, Ca treatments, and blocks on shoot yield (log g) of 2 *Alyssum* spp. and 1 cabbage reference species.

Source	DF	MS	F values
Species	2	6.7144	106.98***
Ca trt	4	4.2034	66.97***
Species x Ca trt	8	0.5064	8.07***
Ni trt	3	8.6326	137.55***
Species x Ni trt	2	0.2012	3.21*
Ni trt x Ca trt	12	0.9326	14.86***
Species x Ni trt x Ca trt	8	0.2782	4.43***
Block	2	0.0207	0.72
Error	78	0.06276	

*, ***, Significant at the probability 0.05 and 0.001 levels, respectively.

Tests of hypotheses using the Type I MS for Species x Ni trt x Ca trt as the error term.

Table1B. Mean squares (MS) for the combined analysis over species, Ni treatments, Ca treatments, and blocks on shoot Ni concentration† of 2 *Alyssum* spp. and 1 cabbage reference species.

Source	DF	MS	F values
Species	2	382.6	5196.***
Ca trt	4	0.7585	10.30***
Species x Ca trt	8	0.2762	3.75***
Ni trt	3	54.8583	744.90***
Species x Ni trt	2	0.0857	1.16
Ni trt x Ca trt	12	0.3281	4.45***
Species x Ni trt x Ca trt	8	0.2235	3.04**
Block	2	0.0634	0.86
Error	78	0.0736	

, *, Significant at the probability 0.01 and 0.001 levels, respectively.

Type I MS for Species x Ni trt x Ca trt was used as the error term to test for hypotheses.

† Ni concentration is log mg L^{-1}.

Table 1C. Mean squares (MS) for the combined analyses by species, Ni treatments, Ca treatments, and blocks on shoot yield† of 2 *Alyssum* spp. and 1 cabbage reference species.

Source	DF	MS	F values
Species: *Alyssum murale*			
Ni trt	2	2.318	47.55***
Ca trt	4	1.084	22.24***
Ni trt x Ca trt	8	0.481	9.86***
Error	30	0.9157	

Source	DF	MS	F values
Species: *Alyssum pintodasilvae*			
Ni trt	2	3.904	44.43***
Ca trt	4	2.399	27.30***
Ni trt x Ca trt	8	0.759	8.64***
Error	30	0.0879	

Source	DF	MS	F values
Species: Cabbage			
Ni trt	1	13.86	330.67
Ca trt	4	1.733	41.35
Ni trt x Ca trt	4	0.8740	20.86
Error	20	0.0419	

*, ***, Significant at the probability 0.05 and 0.001 levels, respectively.

Type III MS for Species x Ni trt x Ca trt was used as the error term to test for hypotheses.

† shoot yield is log g.

Table 1D. Mean squares (MS) for the combined analyses by species, Ni treatments, Ca treatments, and blocks on shoot Ni concentration† of 2 *Alyssum* spp. and 1 cabbage reference species.

Source	DF	MS	F values
Species: *Alyssum murale*			
Ca trt	2	8.957	161.18***
Ni trt	4	0.9013	16.22***
Ni trt x Ca trt	8	0.2965	5.34***
Error	30	0.0556	

Source	DF	MS	F values
Species: *Alyssum pintodasilvae*			
Ca trt	2	6.650	104.87***
Ni trt	4	0.2614	4.12**
Ni trt x Ca trt	8	0.2013	3.17**
Error	30	0.06341	

Source	DF	MS	F values
Species: Cabbage			
Ni trt	1	133.5	1160.34***
Ca trt	4	0.1482	1.29
Ni trt x Ca trt	4	0.4358	3.79*
Error	20	0.1151	

*, ***, Significant at the probability 0.05 and 0.001 levels, respectively.

Type III MS for Species x Ni trt x Ca trt was used as the error term to test for hypotheses.

† Ni concentration is log mg L^{-1}.

Table 2. Nickel concentration for Ni treatment† additions to 0.5 strength Hoagland solution with 2.0 mM $MgSO_4$, respectively.

Treatment	Concentration	Treatment	Concentration
---μM---	—— pNi^{2+} mol/L ——	---μM---	—— pCa^{2+} mol/L ——
—Ni—		—Ca—	
Cabbage			
1.00	6	128	3.89
10.00	5	320	3.49
		800	3.10
		2000	2.70
		5000	2.30
Alyssum spp.‡			
31.60	5.50	128	3.89
316.00	3.50	320	3.49
1000.00	3.00	800	3.10
		2000	2.70
		5000	2.30

† $NiSO_4$ was used as nickel treatments. Due to the death of cabbage before nickel treatment reaching 31.6 mM in pre-experiment, cabbage was only applied 2 lower nickel levels.

‡ *Alyssum* spp. are *Alyssum murale* and *Alyssum pintodasilvae*

Table 3. Mean squares from analysis of variance of shoot, root, and whole plant dry matter yield, Ni concentration, and Ca concentration for *A. murale*, *A. pintodasilvae*, and cabbage across Ca and Ni treatments, respectively.

Source	df	Root	Shoot	Whole Plant
		Dry matter yield		
		log g		
A. murale	14	2.78*	0.92***	0.95***
A. pintodasilvae	14	2.03***	1.68***	1.67***
Cabbage	9	7.30***	2.70***	3.00***
		Ni Concentration		
		log mg kg^{-1}		
A. murale	14	4.03***	1.71***	1.82***
A. pintodasilvae	14	3.21***	1.14***	1.21***
Cabbage	9	13.73***	15.10***	12.42***
		Ca Concentration		
		log mg kg^{-1}		
A. murale	14	0.480+	3.48***	2.95***
A. pintodasilvae	14	0.329***	2.55***	2.31***
Cabbage	9	3.06***	2.73***	2.74***

+, *, *** Significant at the 0.1, 0.05, and 0.001 of probabilities, respectively.

Table 4A. Matrix of correlation coefficient (r) of interelemental relationships in Ni hyperaccumulator, *Alyssum murale*, grown in 0.5 strength Hoagland solution with nickel and calcium treatments.

N	Shoot dry wt.	Zn	Cu	Mn	Fe	P	Mg	Ca
Ni	-0.52***	0.76***	0.98***	-0.02	-0.22	0.51***	-0.13	0.36*
Shoot dry wt.		-0.54***	-0.51***	-0.05	-0.09	-0.84***	-0.26+	-0.07
Zn			0.76***	0.19	-0.06	0.51***	-0.14	0.48***
Cu				-0.03	-0.29+	0.50***	-0.15	0.40**
Mn					0.03	-0.01	-0.19	0.67***
Fe						0.06	0.28+	-0.19
P							0.29+	-0.02
Mg								-0.53***
Ca								

+, *, **, ***Significant at the 0.1, 0.05, 0.01 and 0.001 probability level, respectively.
(Check reference from Brooks and Yang, 1984, Taxon 33(3):392-399. In Ca/Mg paper file)

Table 4B. Matrix of correlation coefficient (r) of interelemental relationships in Ni hyperaccumulator, *Alyssum pintodasilvae*, grown in 0.5 strength Hoagland solution with nickel and calcium treatments.

	Ni	Shoot dry wt.	Zn	Cu	Mn	Fe	P	Mg	Ca
Ni		-0.40**	0.70***	1.00***	-0.57***	-0.62***	0.57***	0.10	0.10
Shoot dry wt.			-0.56***	-0.41**	-0.13	0.42**	-0.73***	-0.12	-0.07
Zn				0.68***	-0.01	-0.32*	0.81***	0.03	0.28+
Cu					-0.57***	-0.63***	0.58***	0.10	0.10
Mn						0.41**	-0.02	-0.11	0.27+
Fe							-0.34*	0.00	-0.17
P								0.19	0.05
Mg									-0.66***
Ca									

+, *, **, ***Significant at the 0.1, 0.05, 0.01 and 0.001 probability level, respectively.
(Check reference from Brooks and Yang, 1984, Taxon 33(3):392-399. In Ca/Mg paper file)

Table C. Matrix of correlation coefficient (r) of interelemental relationships in cabbage grown in 0.5 strength Hoagland solution with nickel and calcium treatments.

	Ni	Shoor dry wt.	Zn	Cu	Mn	Fe	P	Mg	Ca
Ni		-0.76***	0.73***	0.78***	0.55**	-0.16	0.82***	0.31+	-0.11
Shoot dry wt.			-0.74***	-0.74***	-0.68***	-0.14	-0.85***	-0.51**	0.18
Zn				0.87***	0.57***	-0.10	0.89***	0.44*	-0.26
Cu					0.54**	-0.09	0.85***	0.38*	-0.11
Mn						0.37*	0.67***	0.76***	-0.23
Fe							-0.06	0.50**	-0.17
P								0.40**	-0.17
Mg									-0.69***
Ca									

+, *, **, ***Significant at the 0.1, 0.05, 0.01 and 0.001 probability level, respectively.
(Check reference from Brooks and Yang, 1984, Taxon 33(3):392-399. In Ca/Mg paper file)

Table 5A. Elemental concentrations in shoots and roots of *Alyssum murale* grown in nutrient solution with three Ni treatments and five Ca treatments. Geometric means are presented, n=3.

Treatment conc.	Yield	Ni	Zn	Cu	Mn	Fe	P	Mg	Ca
-log mol L^{-1}	g	g kg^{-1} dry wt	mg kg^{-1} dry wt				g kg^{-1} D wt		
Shoot									
pNi = 4.5									
Ca level									
3.89	3.24 b	3.67 a	69.7 a	21.6 a	109. a	95.7 a	3.40 a	5.75 a	2.47 d
3.49	4.16 a	1.69 b	54.4 a	13.6 a	102. a	99.6 a	2.91 a	4.84 ab	5.60 d
3.10	3.56 ab	2.68 ab	63.2 a	21.8 a	128. a	88.2 a	3.35 a	4.87 ab	17.0 c
2.70	3.19 b	2.56 ab	56.1 a	22.1 a	131. a	87.6 a	3.37 a	4.46 bc	24.5 b
2.30	3.19 b	2.51 ab	65.4 a	30.1 a	152. a	56.9 b	3.14 a	3.61 c	39.1 a
pNi = 3.5									
Ca level									
3.89	2.27 b	5.93 c	86.7 ab	51.2 bc	50.9 b	59.0 b	3.87 ab	4.53 ab	1.97 c
3.49	3.74 b	5.77 c	46.2 b	43.2 c	43.7 b	58.2 b	2.61 ab	4.85 a	3.67 c
3.10	3.60 b	8.37 b	80.6 ab	71.8 b	54.6 b	80.8 a	3.74 a	5.43 a	11.2 bc
2.70	5.43 a	4.82 c	55.3 b	40.2 c	37.5 b	75.1 a	1.92 b	3.90 ab	15.6 b
2.30	3.19 b	16.5 a	111.8 a	133.5 a	163. a	43.8 c	3.33 ab	2.79 b	40.0 a
pNi = 3.0									
Ca level									
3.89	0.79 b	13.1 b	80.8 b	98.0 b	44.7 b	64.0 a	6.91 a	5.66 a	2.03 c
3.49	1.48 b	9.23 b	63.8 b	77.6 b	40.8 b	86.7 a	4.91 a	5.61 a	3.98 c
3.10	3.78 a	8.58 b	56.2 b	69.7 b	28.4 b	57.0 a	2.62 b	4.72 ab	5.86 c
2.70	3.98 a	8.18 b	71.1 b	63.5 b	22.2 b	62.4 a	2.78 b	3.25 b	11.2 b
2.30	0.96 b	22.8 a	203. a	169.8 a	86.4 a	84.4 a	5.50 a	3.93 ab	37.0 a

Root									
pNi = 4.5									
Ca level									
3.89	0.518 a	0.351 a	223. a	20.7 a	136. a	361 a	3.01 a	1.99 b	1.93 d
3.49	0.560 a	0.411 a	65.3 a	27.3 a	137. a	288 a	2.31 a	2.19 b	2.19cd
3.10	1.08 a	0.451 a	166. a	11.3 a	162. a	187 a	3.05 a	1.57 b	2.81 c
2.70	1.05 a	0.466 a	163. a	15.6 a	147. a	180 a	3.99 a	1.59 b	3.79 b
2.30	0.625 a	1.15 a	264. a	16.2 a	467. a	313 a	4.79 a	3.20 a	5.31 a
pNi = 3.5									
Ca level									
3.89	0.641 b	2.09 b	439. a	60.1 a	41.5 b	2640 a	5.06 a	2.27 ab	1.73 c
3.49	0.886 ab	2.25 b	199. b	28.3 b	19.0 b	939 b	3.81 ab	1.94 ab	1.72 c
3.10	0.800 ab	2.02 b	212. b	29.3 b	26.6 b	1230 b	3.68 ab	1.74 ab	2.30 c
2.70	1.63 a	2.22 b	83.9 b	17.3 b	20.1 b	701 b	2.05 b	1.50 b	3.46 b
2.30	1.98 ab	7.90 a	155. b	60.1 a	196. a	430 b	3.38 b	2.45 a	4.48 q
pNi = 3.0									
Ca level									
3.89	0.0682 a	8.17 ab	320. ab	86.0 a	58.7 a	1756 a	10.5 a	5.78 a	3.96 a
3.49	0.0576 a	4.49 b	262. ab	159.0 a	88.7 a	2330 a	7.92 a	4.97 a	4.26 a
3.10	0.732 a	5.23 b	136. b	48.2 a	24.1 a	1429 a	3.50 a	1.67 a	2.01 a
2.70	0.432 a	3.86 b	122. b	50.3 a	28.7 a	2595 a	4.16 a	1.58 a	3.95 a
2.30	0.372 a	10.5 a	441. a	83.8 a	44.1 a	2237 a	7.61 a	2.83 a	5.04 a

‡Means followed by the same letter across treatments and within a plant part are not significantly different (P=0.05, df =10).

Table 5B. Elemental concentrations in shoots and roots of *Alyssum pintodasilvae* grown in nutrient solution with three Ni treatments and five Ca treatments. Geometric means are presented, n=3.

Treatment conc.	Yield	Ni	Zn	Cu	Mn	Fe	P	Mg	Ca
-log mol L^{-1}	g	mg kg^{-1} D. wt	mg kg^{-1} D. wt				g kg^{-1} D. wt		
Shoot									
pNi = 4.5									
Ca level									
3.89	1.69 b	4.60 a	120. a	36.7 a	198. a	82.1 ab	5.34 a	5.55 a	5.31 d
3.49	2.01 b	3.36 ab	84.0 a	25.5 ab	189. a	109. ab	3.94 a	5.06 ab	9.99 d
3.10	3.75 a	2.02 b	62.4 a	15.5 b	166. a	119. a	3.33 a	4.34 b	19.2 c
2.70	2.60 ab	3.22 ab	86.3 a	26.8 ab	181. a	94.9 ab	4.17 a	4.04 bc	40.2 b
2.30	2.12 b	3.77 ab	95.3 a	32.1 ab	236. a	61.3 b	4.00 a	3.08 c	51.8 a
pNi = 3.5									
Ca level									
3.89	2.10 b	9.16 ab	120. a	68.3 ab	97.3 ab	73.5 a	4.83 a	5.30 a	3.64 d
3.49	4.00 a	8.37 ab	87.9 a	64.5 ab	78.4 abc	59.8 ab	3.22 b	5.60 a	6.64 cd
3.10	4.29 a	6.81 b	80.7 a	52.4 b	57.4 c	76.6 a	2.85 b	4.70 ab	12.6 c
2.70	3.64 ab	6.96 b	83.8 a	56.5 b	63.8 c	74.9 a	3.08 b	4.33 ab	29.9 b
2.30	2.16 b	10.8 a	112. a	83.5 a	102. a	50.2 b	3.80 ab	3.35 b	55.6 a
pNi = 3.0									
Ca level									
3.89	0.229 d	7.76 c	75.4 b	63.2 c	55.4 a	20.7 c	4.75 ab	4.92 a	6.83 d
3.49	1.26 bc	13.6 b	136. b	110. b	53.1 a	48.4 b	6.79 a	5.59 a	5.49 d
3.10	1.74 b	13.3 b	119. b	106. ab	53.4 a	64.9 a	5.46 ab	5.10 a	12.9 c
2.70	4.02 a	10.1 c	85.6 b	79.4 c	34.6 a	65.4 a	3.19 b	4.10 a	19.3 b
2.30	0.989 c	16.9 a	208. a	131. a	63.3 a	28.1 c	7.50 a	3.88 a	50.3 a

Root									
pNi = 3.5									
Ca level									
3.89	0.231 a	1.050 a	658. a	20.9 a	152. b	1110. a	4.48 a	2.25 a	1.78 c
3.49	0.393 a	0.461 b	298. a	13.8 a	120. b	437. a	2.83 a	1.59 b	2.20 bc
3.10	0.412 a	0.371 b	266. a	13.5 a	48.1 b	529. a	3.62 a	1.61 b	3.14 ab
2.70	0.483 a	0.473 b	260. a	14.2 a	110. b	401. a	2.97 a	1.25 b	3.51 ab
2.30	0.503 a	0.593 ab	290. a	14.2 a	472. a	378. a	3.99 a	1.56 b	4.69 a
pNi = 3.5									
Ca level									
3.89	0.282 b	2.10 a	354. a	32.1 a	41.9 a	2880 a	3.19 a	2.15 a	2.15 c
3.49	0.512 ab	1.94 a	230. ab	27.7 a	32.6 a	1860 a	3.74 a	1.90 a	1.81 c
3.10	0.804 a	1.32 a	117. b	24.0 a	28.8 a	1240 a	3.21 a	1.54 a	2.29 c
2.70	0.545 ab	1.17 a	150. ab	22.4 a	25.2 a	2040 a	3.19 a	1.36 a	3.10 b
2.30	0.511 ab	2.90 a	298. ab	31.6 a	35.3 a	1440 a	3.74 a	1.70 a	4.33 a
pNi = 3.0									
Ca level									
3.89	0.027 c	7.95 a	212. b	236. a	65.1 a	2620 a	7.13 a	7.48 a	4.06 a
3.49	0.124 bc	6.28 ab	705. a	73.1 b	39.1 b	3820 a	6.50 a	2.16 b	2.43 a
3.10	0.248 ab	4.83 bc	487. a	39.7 b	32.2 b	4180 a	4.98 ab	1.88 b	2.59 a
2.70	0.463 a	2.58 c	199. b	25.2 b	22.5 b	2140 a	2.97 b	1.38 b	2.95 a
2.30	0.225 bc	7.26 ab	626. a	70.0 b	31.5 b	2120 a	7.87 a	1.71 b	4.82 a

‡Means followed by the same letter across treatments and within a plant part are not significantly different (P=0.05, df =10).

Table 5C. Elemental concentrations in shoots and roots of cabbage grown in nutrient solution with three Ni treatments and five Ca treatments. Geometric means are presented, n=3.

Treatment conc.	Yield	Ni	Zn	Cu	Mn	Fe	P	Mg	Ca
-log mol L^{-1}	g	g kg^{-1} D. wt	----------	mg kg^{-1} D. wt	----------	----------	g kg^{-1} D. wt	--------	
Shoot									
pNi = 6.0									
Ca level									
3.89	4.72 c	0.00215 a	56.4 a	2.58 a	133. a	74.3 a	2.10 a	16.1 a	3.39 d
3.49	9.49 b	0.00152 a	20.3 b	1.92 a	58.7 b	41.6 ab	0.96 b	9.06 bc	3.45 d
3.10	11.1 ab	0.00297 a	24.4 b	2.09 a	75.6 b	49.4 ab	0.93 b	10.7 b	10.6 c
2.70	12.6 a	0.00211 a	22.1 b	2.08 a	70.6 b	32.5 b	0.80 b	6.63 cd	19.8 b
2.30	12.2 a	0.00305 a	20.8 b	2.23 a	63.6 b	42.7 ab	0.86 b	5.19 d	34.2 a
pNi = 5.0									
Ca level									
3.89	1.62 bc	0.234 a	219. a	6.03 a	132. a	38.0 a	6.09 a	15.2 a	3.27 d
3.49	1.87 bc	0.193 ab	237. a	4.34 ab	134. a	50.7 a	5.92 a	12.6 b	7.56 cd
3.10	1.13 c	0.162 bc	209. a	6.05 a	103. ab	44.6 a	7.32 a	9.64 c	14.8 bc
2.70	9.98 a	0.116 c	16.3 b	2.75 b	62.9 b	38.9 a	0.75 b	6.53 d	19.9 b
2.30	2.51 b	0.106 c	83.2 ab	3.54 ab	98.0 ab	35.7 a	3.75 ab	6.43 d	41.7 a
Root									
pNi = 6.0									
Ca level									
3.89	1.11 b	0.0113 a	25.8 a	14.8 a	42.1 a	130. a	1.98 a	3.70 b	1.39 b
3.49	1.89 ab	0.0171 a	30.3 a	17.2 a	31.6 a	143. a	1.96 a	3.55 b	1.47 b
3.10	2.64 a	0.0144 a	18.2 a	8.00 a	21.5 a	106. a	1.24 a	3.51 b	1.80 b
2.70	2.77 a	0.0183 a	17.5 a	9.43 a	20.4 a	90.9 a	1.33 a	3.82 b	3.17 b
2.30	2.39 a	0.0145 a	20.0 a	9.53 a	39.6 a	98.7 a	1.57 a	4.85 a	42.0 a

pNi = 5.0									
Ca level									
3.89	0.120 b	1.05 a	249. a	64.1 a	121. a	836. ab	6.56 ab	4.33 ab	3.90 b
3.49	0.077 b	1.08 a	326. a	47.0 a	94.1 a	1370. ab	5.97 ab	3.45 b	4.08 ab
3.10	0.045 b	0.733 a	546. a	52.3 a	121. a	2520. a	11.2 a	5.47 a	5.61 ab
2.70	0.893 a	0.408 a	61.6 a	21.7 a	38.7 a	281. b	2.32 b	2.65 b	4.09 ab
2.30	0.409 ab	0.996 a	141. a	25.7 a	213. a	367. b	3.31 b	2.97 b	9.48 a

‡Means followed by the same letter across treatments and within a plant part are not significantly different (P=0.05, df =10).

CLAIMS:

1. A method of recovering nickel from soil rich in nickel, comprising:

growing a nickel hyperaccumulating plant selected from the genera *Alyssum* on said soil, while maintaining soil conditions such that the concentration of calcium in said soil is from about 0.128 mM to about 5 mM and said pH is maintained below about 7.0,

Allowing said growth to continue until such time as the concentration of Ni in the above ground tissues of said plant is at least 2.5%, gross dry weight of the above ground tissues,

drying said above ground tissues, and

recovering Ni from said above ground tissues.

2. The method of claim 1, wherein said above ground tissues are selected from the group consisting of shoots, leaves, above ground tissues other than shoots and leaves, and mixtures thereof.

3. The method of claim 2, wherein said above ground tissues are leaves.

4. The method of claim 1, wherein said soil conditions are maintained such that the ratio of exchangeable Ca/Mg is between about 0.16 - 0.40.

5. The method of claim 1, wherein said plant is selected from a species selected from the group consisting of *A. murale, A. pintodasilvae, A. malacitanum, A. lesbiacum, A. tenium,* and *A. fallacinum.*

6. The method of claim 5, wherein said plant is selected from a species selected from the group consisting of *A. murale* and *A. pintodasilvae.*

7. The method of claim 1, wherein the genotype of said plant is identical to that of the wild-type of said species and free of natural or induced mutation and heterologous DNA.

8. The method of claim 1, wherein said soil conditions are further maintained such that chelating agents which chelate Ni in the presence of Fe, Mg

and Ca are added to said soil and ammonium based N-fertilizer is added to said soil, both while said plant is being grown on said soil.

9. The method of claim 1, wherein said soil is serpentine soil.

10. The method of claim 1, wherein said soil is rich in Ni due to at least one industrial process which has deposited Ni in said soil.

11. A naturally occurring plant of the *Allysum* genus which has a concentration of nickel in its above-ground tissues of 2.5 - 5.0%, based on the gross dry weight of said tissues.

FIGURE 1

Cabbage - Shoot Yield

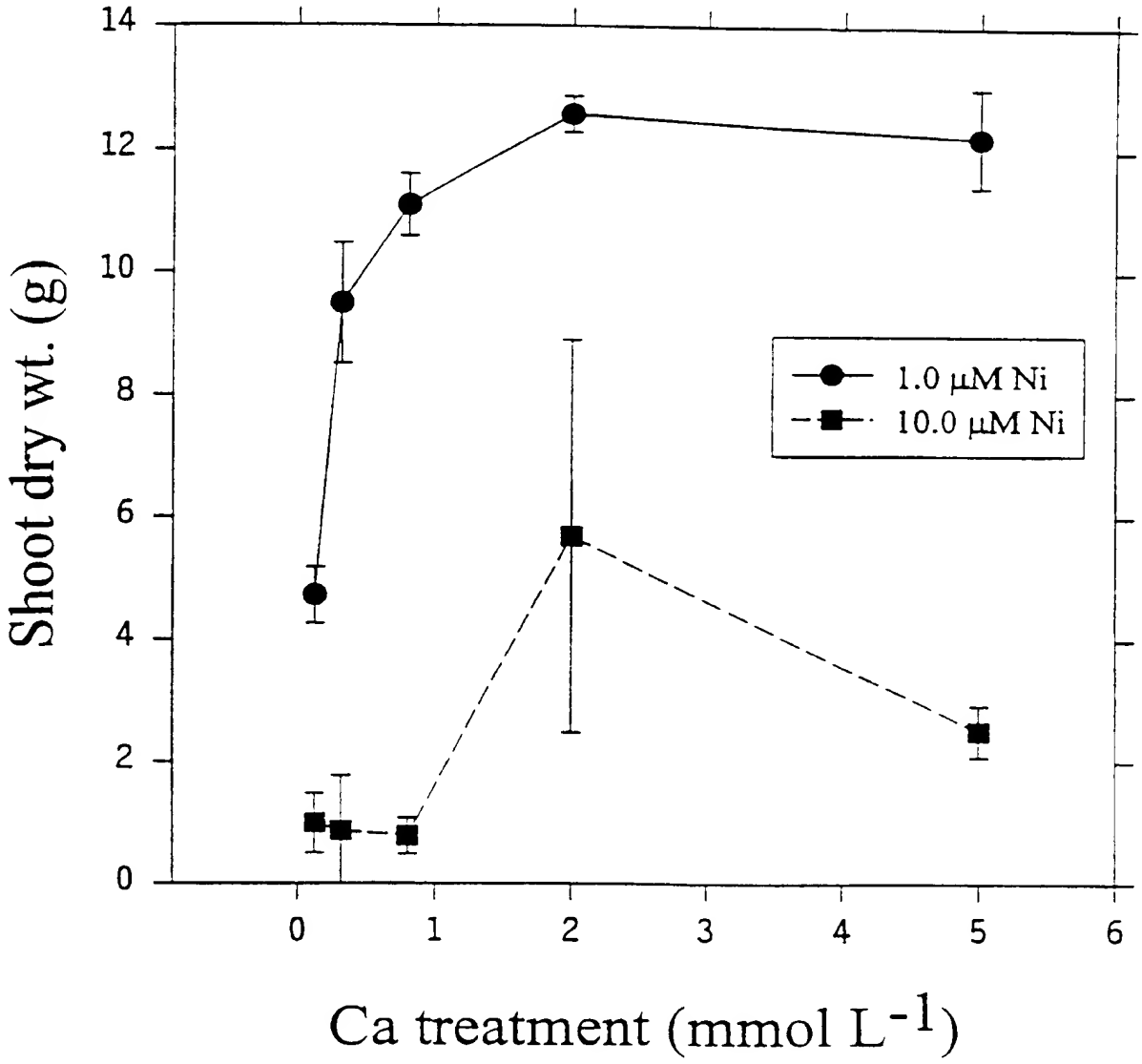


FIGURE 2

A. murale Shoot Yield

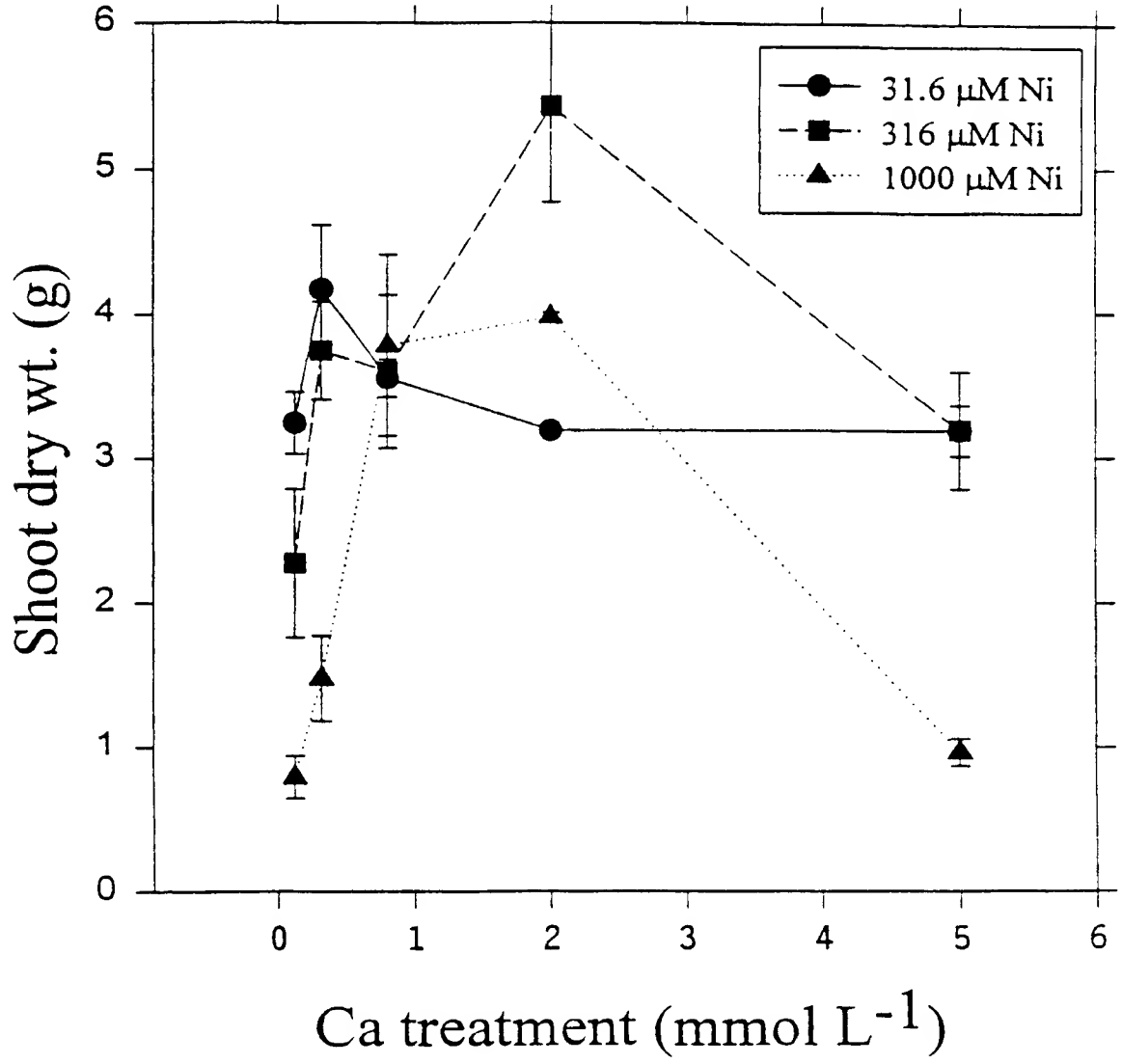


FIGURE 3

A. pintodasilvae Shoot Yield

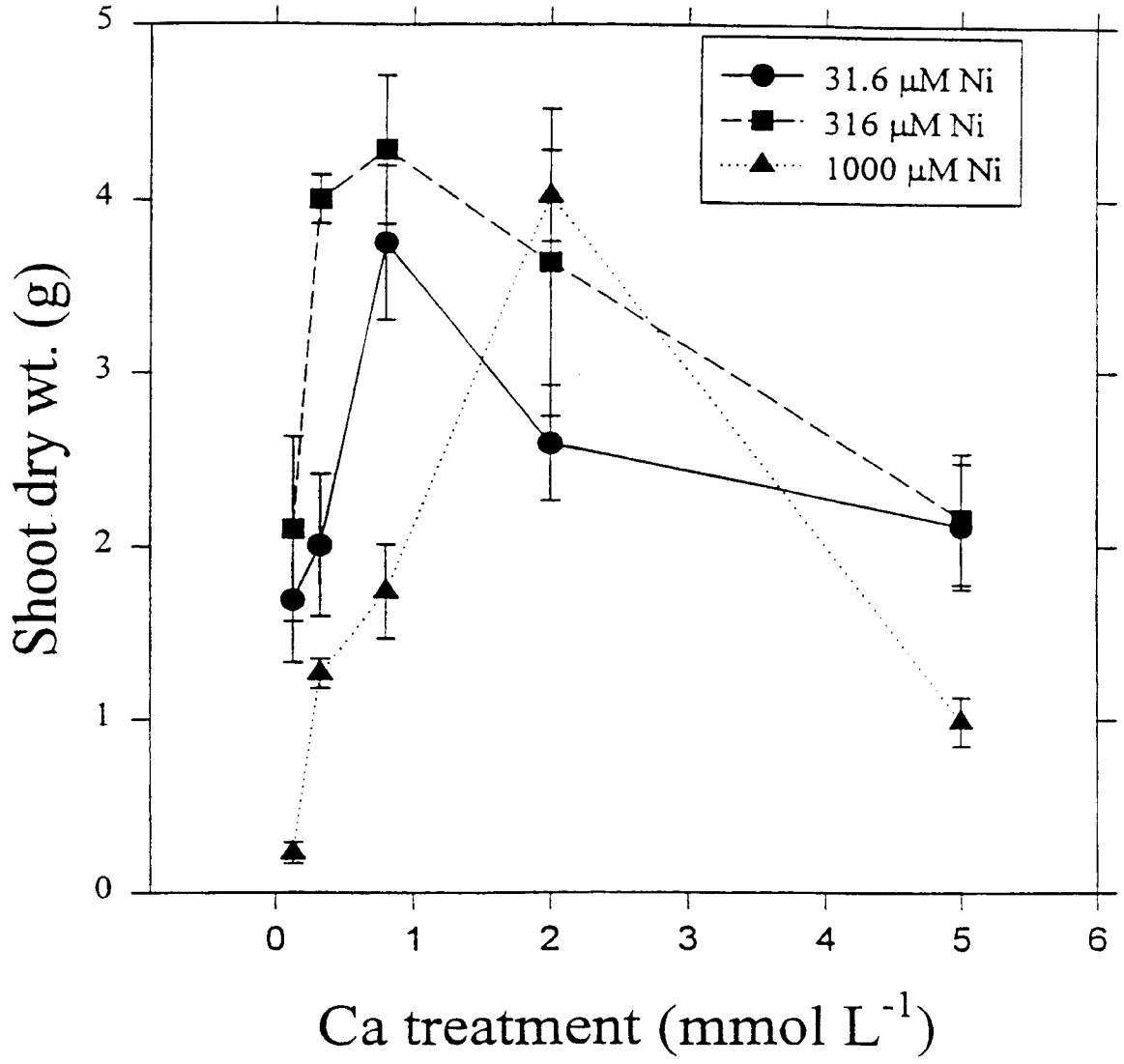


FIGURE 4

Cabbage - Shoot Ni Concentration

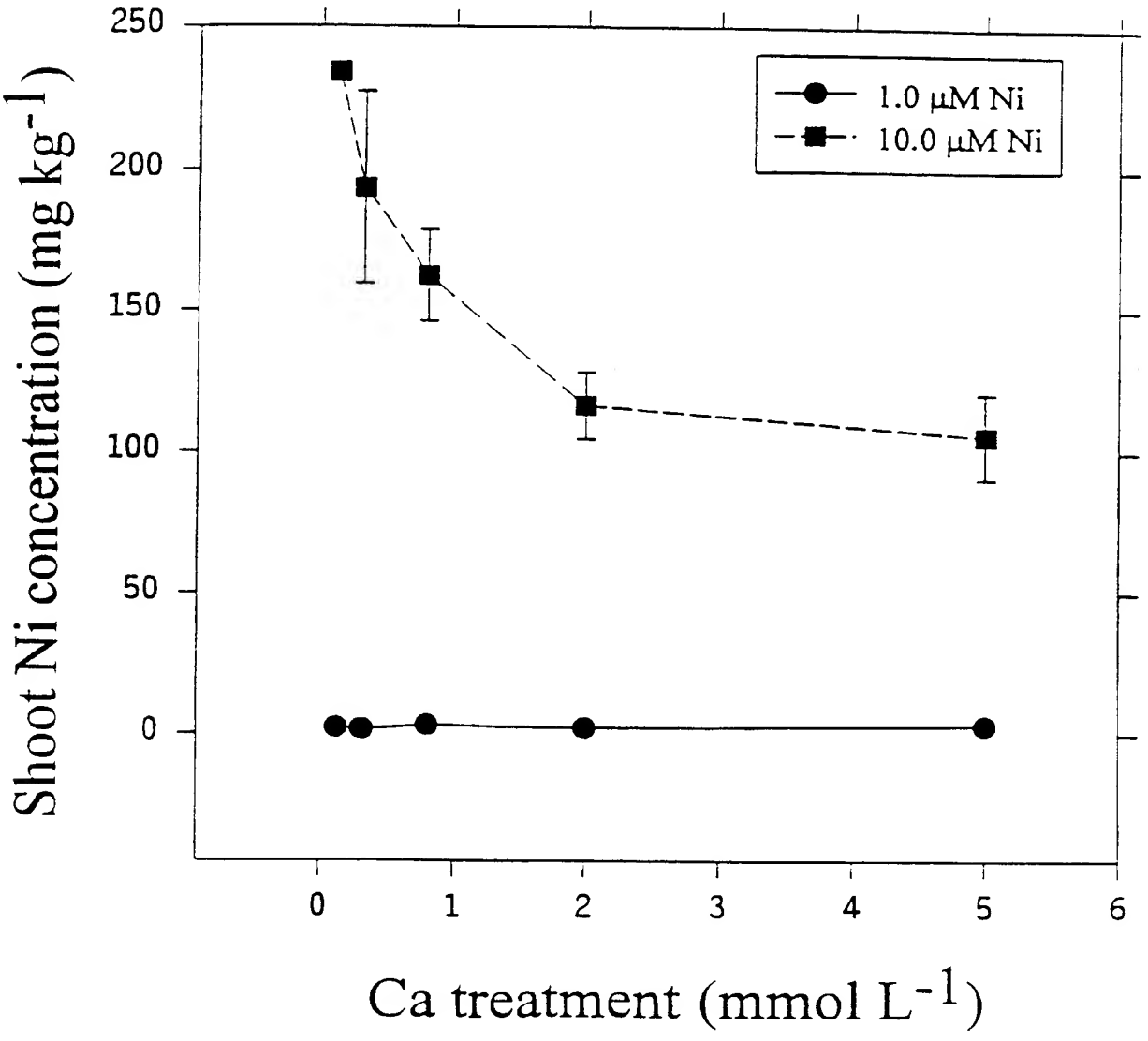


FIGURE 5

A. murale Shoot Ni Concentration

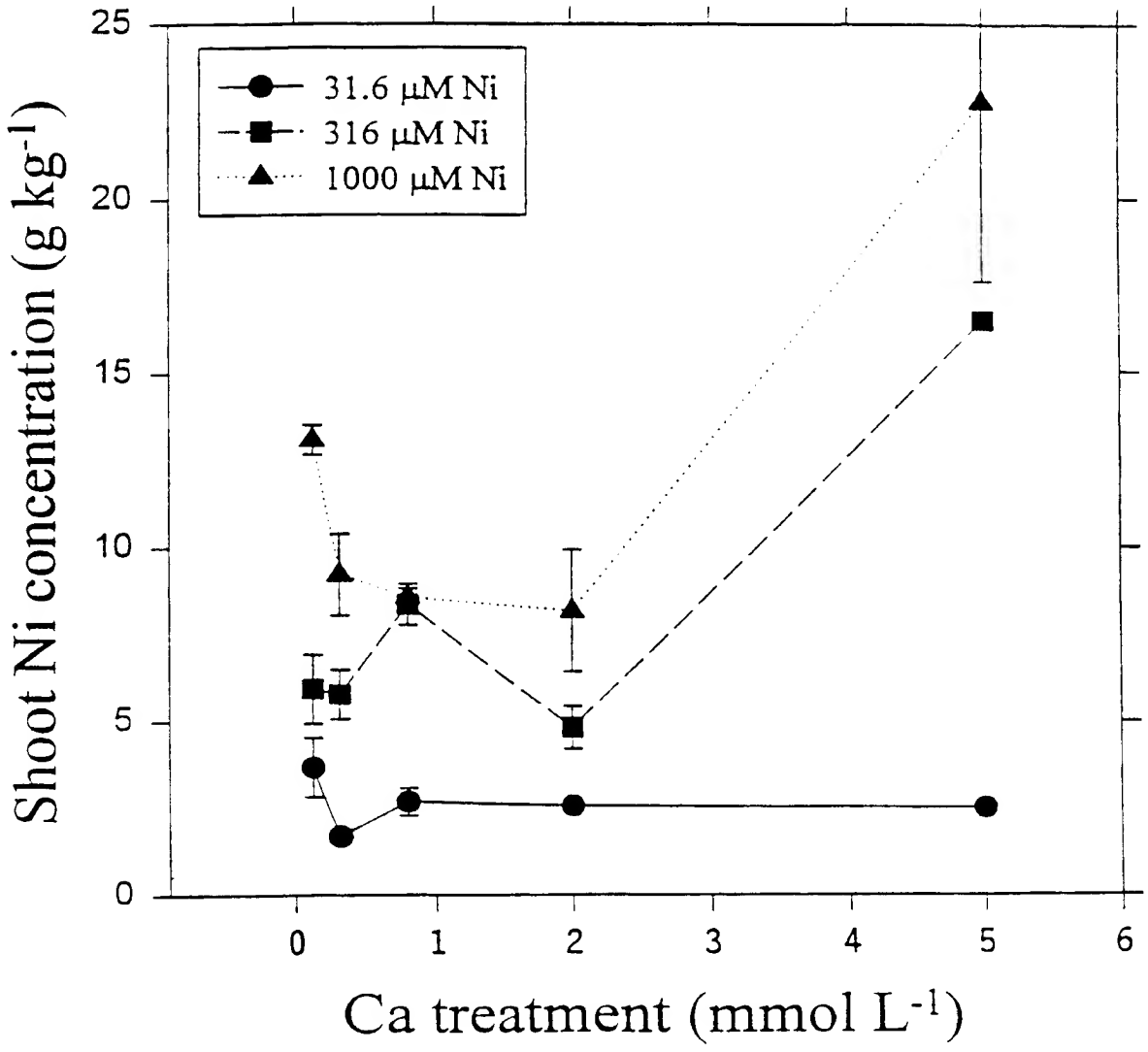


FIGURE 6

A. pintodasilvae Shoot Ni Concentration

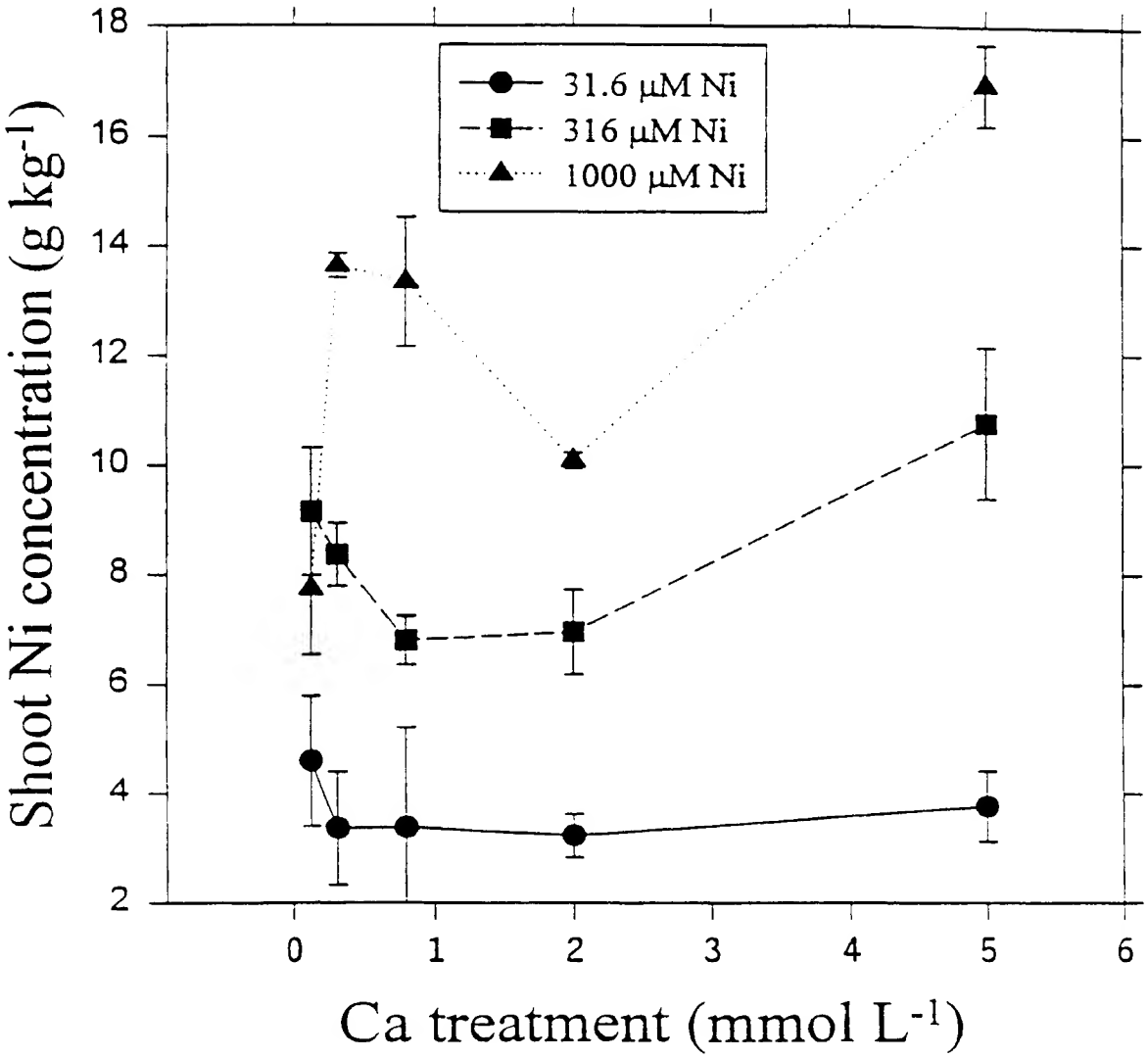


FIGURE 7

A. murale Shoot: Root Ni Ratio

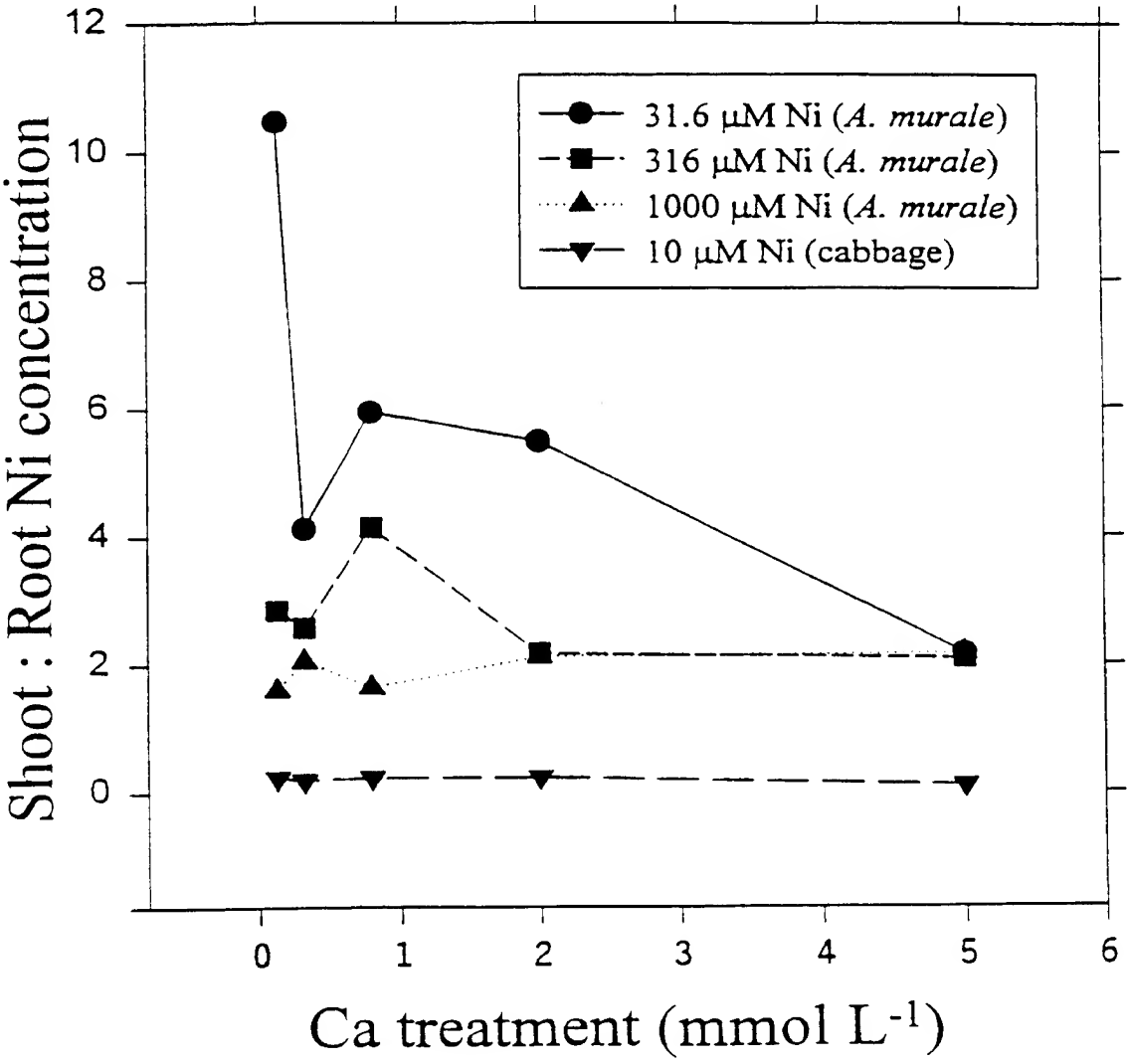


FIGURE 8

A. pintodasilvae Shoot: Root Ni Ratio

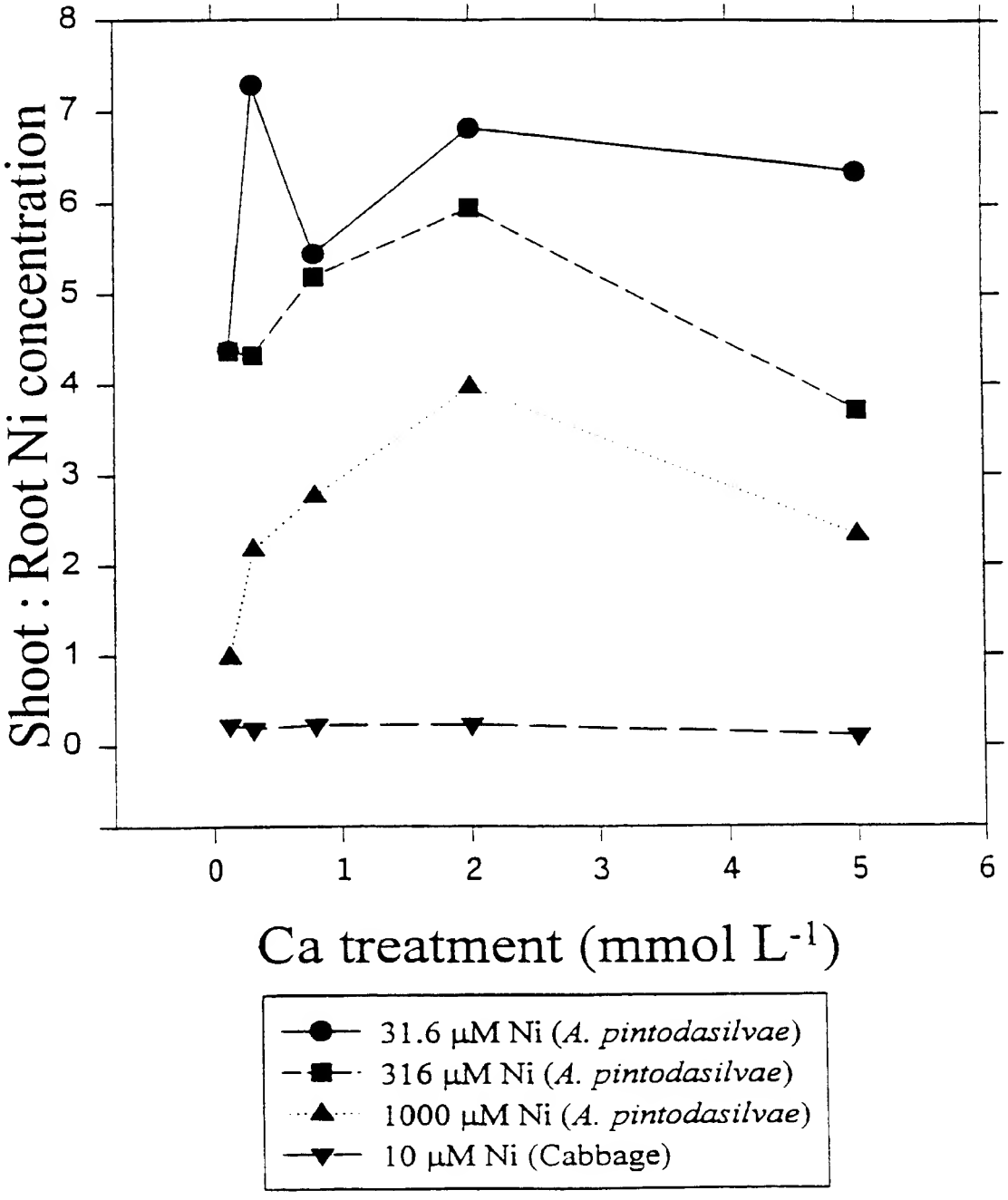


FIGURE 9

A. murale Shoot Ni Content

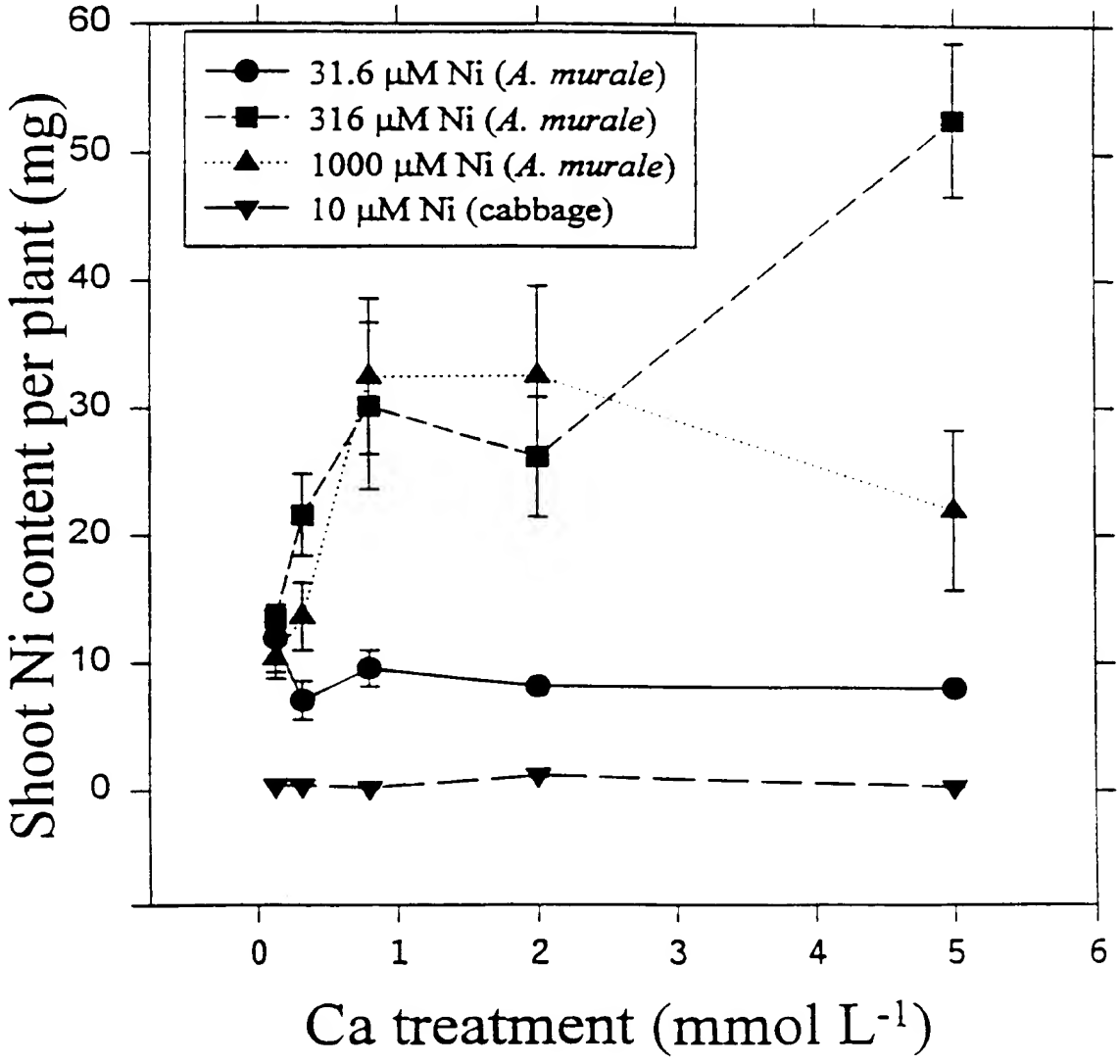
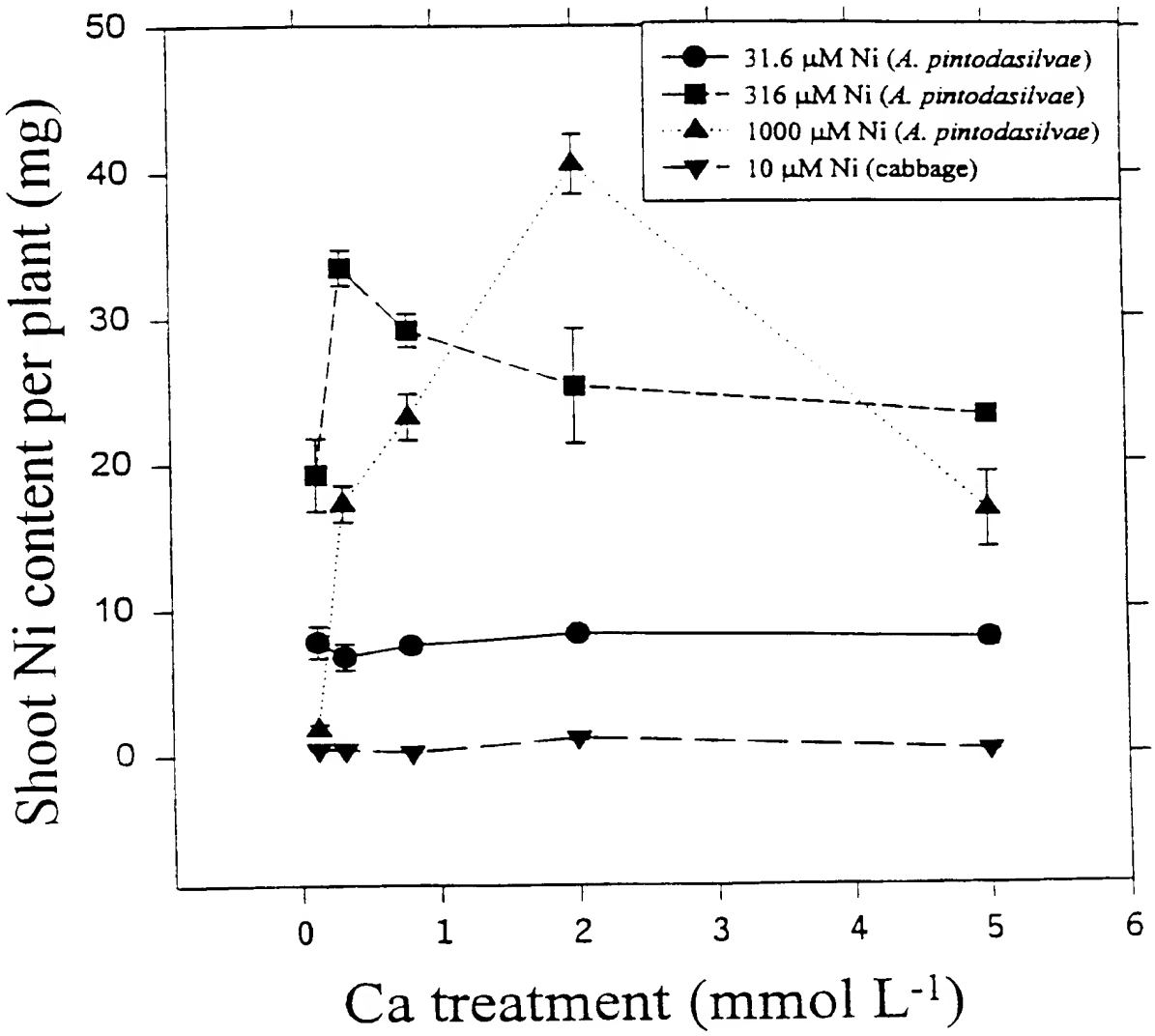


FIGURE 10

A. pintodasilvae Shoot Ni Content



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/15109

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :C22B 23/00
US CL :75/710
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 75/710, 392, 432; 210/602, 682, 688; 71/9

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,364,451 (RASKIN et al.) 15 November 1994, col. 1, lines 27-60.	1 & 11

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
02 DECEMBER 1997

Date of mailing of the international search report
27 JAN 1998

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer
M. ALEXANDRA ELVE
Telephone No. (703) 308-0092

Form PCT/ISA/210 (second sheet)(July 1992)★

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To: STEVEN B. KELBER
OBLON, SPIVAK, MCCLELLAND, MAIER & NEUSTADT
1755 JEFFERSON DAVIS HWY.
CRYSTAL SQUARE 5, 4TH FL.
ARLINGTON, VA 22202

PCT

NOTIFICATION OF TRANSMITTAL OF INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

Date of Mailing *(day/month/year)*	09 OCT 1998
Applicant's or agent's file reference 274/091£7CIP PCT	IMPORTANT NOTIFICATION

International application No.	International filing date *(day/month/year)*	Priority Date *(day/month/year)*
PCT/US97/15109	29 AUGUST 1997	30 AUGUST 1996

Applicant
CHANEY, RUFUS L.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. REMINDER

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices)(Article 39(1))(see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer M. ALEXANDRA ELVE
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0661

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 274709127CIP	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/US97/15109	International filing date *(day/month/year)* 29 AUGUST 1997	Priority date *(day/month/year)* 30 AUGUST 1996
International Patent Classification (IPC) or national classification and IPC IPC(6): C22B 23/00 and US Cl.: 75/710		
Applicant CHANEY, RUFUS L.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 23 sheets.

☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of 11 sheets.

3. This report contains indications relating to the following items:

- I [X] Basis of the report
- II [ ] Priority
- III [ ] Non-establishment of report with regard to novelty, inventive step or industrial applicability
- IV [ ] Lack of unity of invention
- V [X] Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI [ ] Certain documents cited
- VII [ ] Certain defects in the international application
- VIII [ ] Certain observations on the international application

Date of submission of the demand	Date of completion of this report
19 FEBRUARY 1998	07 AUGUST 1998
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer M. ALEXANDRA ELVE
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0661

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US97/15109

I. Basis of the report

1. This report has been drawn on the basis of *(Substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments)*:

[X] the international application as originally filed.

[X] the description, pages 1-35 , as originally filed.
pages NONE , filed with the demand.
pages NONE , filed with the letter of _____.
pages _____ , filed with the letter of _____.

[X] the claims, Nos. 1-11 , as originally filed.
Nos. NONE , as amended under Article 19.
Nos. NONE , filed with the demand.
Nos. NONE , filed with the letter of _____.
Nos. _____ , filed with the letter of _____.

[X] the drawings, sheets/~~fig~~ 1-10 , as originally filed.
sheets/~~fig~~ NONE , filed with the demand.
sheets/~~fig~~ NONE , filed with the letter of _____.
sheets/~~fig~~ _____ , filed with the letter of _____.

2. The amendments have resulted in the cancellation of:

[X] the description, pages NONE .
[X] the claims, Nos. NONE .
[X] the drawings, sheets/~~fig~~ NONE .

3. [ ] This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the ~~Supplemental Box~~ Additional observations below (Rule 70.2(c)).

4. Additional observations, if necessary:

NONE

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US97/15109

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. STATEMENT

Novelty (N)	Claims	NONE	YES
	Claims	1-11	NO
Inventive Step (IS)	Claims	NONE	YES
	Claims	1-11	NO
Industrial Applicability (IA)	Claims	1-11	YES
	Claims	NONE	NO

2. CITATIONS AND EXPLANATIONS

Claims 1-11 lack novelty under PCT Article 33(2) as being anticipated by Raskin et al. (US Pat. 5,364,451). Claims 1-11 lack an inventive step under PCT Article 33(3) as being obvious over Raskin et al. Metal ions are removed from the soil by the plant family Brassicaceae which absorbs metals into their roots. Absorbed metals are then transferred to the plant shoots which are harvested (abstract). Plants accumulate metal content of approximately 30% dry weight of plant root and 3.5% dry weight of plant shoot (col. 1, lines 45-52). Metals can include Hg, Cd, Co, Ni, Mo, Cu, As, Se, Zn, Sb, Be, Au, Ba, Mn, Ag, Tl, Rb, Sr, Y, Tc, Ru, Pd, Ir, V, CS, U, Pu, Ce, Pb & Cr (col. 1, lines 52-60). Preferred plant members are Brassica species selected from the group consisting of B. juncea and B. oleracea (col. 2, lines 7-9). Claim 1-11 meet the criteria set out in PCT Article 33(4), because methods of methods of accumulating metals from soils into shoots of a plant have industrial applicability.

NEW CITATIONS

NONE

[redacted]ENT COOPERATION TRE[redacted]

From the INTERNATIONAL BUREAU

PCT

INFORMATION CONCERNING ELECTED OFFICES NOTIFIED OF THEIR ELECTION

PCT Rule 61.3

To:

KELBER, Steven, B.
Oblon, Spivak, McClelland, Maier & Neustadt, P.C.
Crystal Square Five, 4th floor
1755 Jefferson Davis Highway
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

Date of mailing (day/month/year)
27 March 1998 (27.03.98)

Applicant's or agent's file reference
274709127CIP PCT

IMPORTANT INFORMATION

International application No.	**International filing date** (day/month/year)	**Priority date** (day/month/year)
PCT/US97/15109	29 August 1997 (29.08.97)	30 August 1996 (30.08.96)

Applicant
CHANEY, Rufus, L. et al

1. The applicant is hereby informed that the International Bureau has, according to Article 31(7), notified each of the following Offices of its election:

   AP :GH,KE,LS,MW,SD,SZ,UG,ZW
   EP :AT,BE,CH,DE,DK,ES,FI,FR,GB,GR,IE,IT,LU,MC,NL,PT,SE
   National :AU,BG,BR,CA,CN,CZ,DE,FI,GB,IL,JP,KP,KR,MN,NO,NZ,PL,RO,RU,SE,SK, US,VN

2. The following Offices have waived the requirement for the notification of their election; the notification will be sent to them by the International Bureau only upon their request:

   EA :AM,AZ,BY,KG,KZ,MD,RU,TJ,TM
   OA :BF,BJ,CF,CG,CI,CM,GA,GN,ML,MR,NE,SN,TD,TG
   National :AL,AM,AT,AZ,BA,BB,BY,CH,CU,DK,EE,ES,GE,GH,HU,IS,KE,KG,KZ,LC,LK, LR,LS,LT,LU,LV,MD,MG,MK,MW,MX,PT,SD,SG,SI,SL,TJ,TM,TR,TT,UA,UG,UZ,YU,ZW

3. The applicant is reminded that he must enter the "national phase" **before the expiration of 30 months from the priority date** before each of the Offices listed above. This must be done by paying the national fee(s) and furnishing, if prescribed, a translation of the international application (Article 39(1)(a)), as well as, where applicable, by furnishing a translation of any annexes of the international preliminary examination report (Article 36(3)(b) and Rule 74.1).

   Some offices have fixed time limits expiring later than the above-mentioned time limit. For detailed information about the applicable time limits and the acts to be performed upon entry into the national phase before a particular Office, see Volume II of the PCT Applicant's Guide.

   The entry into the European regional phase is postponed **until 31 months from the priority date** for all States designated for the purposes of obtaining a European patent.

RECEIVED
APR 0 3 1998
OBLON, SPIVAK, McCLELLAND
MAIER & NEUSTADT, P.C.

RECEIVED IN FOREIGN FILING
DATE: [redacted]
TIME: [redacted]

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Authorized officer:
R. Raissi

Facsimile No. +41-22-740.14.35

Telephone No. +41-22-338.83.38

PCT / IB/332

The demand must be filed directly with the competent International Preliminary Examining Authority or, if two or more Authorities c with the one chosen by the applicant. The full name or two-letter code of that Authority may be indicated by the applicant on the li

IPEA/ United States

PCT

CHAPTER II

DEMAND

under Article 31 of the Patent Cooperation Treaty:
The undersigned requests that the international application specified below be the subject of international preliminary examination according to the Patent Cooperation Treaty.

For International Preliminary Examining Authority use only

Identification of IPEA	Date of receipt of DEMAND

Box No. I IDENTIFICATION OF THE INTERNATIONAL APPLICATION

Applicant's or agent's file reference: 274709127CIP

International application No.	International filing date *(day/month/year)*	(Earliest) Priority date *(day/month/year)*
PCT/US97/15109	29 August 1997 ( )	30 August 1996 ( )

Title of invention
METHOD FOR PHYTOMINING OF NICKEL, COBALT AND OTHER METALS FROM SOIL

Box No. II APPLICANT(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

CHANEY, Rufus L.
United States Department of Agriculture
Beltsville, Maryland 20705
US

Telephone No.:
Facsimile No.:
Teleprinter No.:

State *(i.e. country)* of nationality:	State *(i.e. country)* of residence:
US	US

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

ANGLE, Jay Scott
10241 Bristol Channel
Ellicot City, Maryland 21042
US

State *(i.e. country)* of nationality:	State *(i.e. country)* of residence:
US	US

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

LI, Yin-Ming
12019 Coldstream Drive
Potomac, Maryland 20854
US

State *(i.e. country)* of nationality:	State *(i.e. country)* of residence:
CN	US

☐ Further applicants are indicated on a continuation sheet.

International application No.
PCT/US97/15109

Box No. III AGENT OR COMMON REPRESENTATIVE: OR ADDRESS FOR CORRESPONDENCE

The following person is ☒ agent ☐ common representative

and ☒ has been appointed earlier and represents the applicant(s) also for international preliminary examination.

☐ is hereby appointed and any earlier appointment of (an) agent(s)/common representative is hereby revoked.

☐ is hereby appointed, specifically for the procedure before the International Preliminary Examining Authority, in addition to the agent(s)/common representative appointed earlier.

Name and address: *(Family name followed by given name; for a legal entity, full official The address must include postal code and name of country.)*

KELBER, Steven B.
OBLON, SPIVAK, McCLELLAND, MAIER & NEUSTADT, P.C.
Crystal Square Five, Fourth Floor
1755 Jefferson Davis Highway
Arlington, Virginia 22202
United States of America

Telephone No.: 703-413-3000

Facsimile No.: 703-413-2220

Teleprinter No.:

☐ Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Box No. IV STATEMENT CONCERNING AMENDMENTS

The applicant wishes the International Preliminary Examining Authority*

(i) ☒ to start the international preliminary examination on the basis of the international application as originally filed.

(ii) ☐ to take into account the amendments under Article 34 of

☐ the description (amendments attached).

☐ the claims (amendments attached).

☐ the drawings (amendments attached).

(iii) ☐ to take into account any amendments of the claims under Article 19 filed with the International Bureau (a copy is attached).

(iv) ☐ to disregard any amendments of the claims made under Article 19 and to consider them as reversed.

(v) ☐ to postpone the start of the international preliminary examination until the expiration of 20 months from the priority date unless that Authority receives a copy of any amendments made under Article 19 or a notice from the applicant that he does not wish to make such amendments (Rule 69.1(d)). *(This check-box may be marked only where the time limit under Article 19 has not yet expired.)*

* Where no check-box is marked, international preliminary examination will start on the basis of the international application as originally filed or, where a copy of amendments to the claims under Article 19 and/or amendments of the international application under Article 34 are received by the International Preliminary Examining Authority before it has begun to draw up a written opinion or the international preliminary examination report, as so amended.

Box No. V ELECTION OF STATES

☒ The applicant hereby elects all eligible States *(that is, all States which have been designated and which are bound by Chapter II of the PCT)* except.....

.....

.....

.....

(If the applicant does not wish to elect certain eligible States, the name(s) or country code(s) of those States must be indicated above.)

See Notes to the demand form

International application No.
PCT/US97/15109

Box No. VI CHECK LIST

The demand is accompanied by the following documents for the purposes of international preliminary examination:		For International Preliminary Examining Authority use only: received	not received
1. amendments under Article 34			
description	sheets	☐	☐
claims	sheets	☐	☐
drawings	sheets	☐	☐
2. letter accompanying amendments under Article 34	sheets	☐	☐
3. copy of amendments under Article 19	sheets	☐	☐
4. copy of statement under Article 19	sheets	☐	☐
5. other *(specify)*:	sheets	☐	☐

The demand is also accompanied by the item(s) marked below:

1. ☐ separate signed power of attorney
2. ☐ copy of general power of attorney
3. ☐ statement explaining lack of signature
4. ☒ fee calculation sheet
5. ☐ other *(specify)*:

Box No. VII SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the demand).

William E. Beaumont

For International Preliminary Examining Authority use only

1. Date of actual receipt of DEMAND:

2. Adjusted date of receipt of demand due to CORRECTIONS under Rule 60.1(b):

3. ☐ The date of receipt of the demand is AFTER the expiration of 19 months from the priority date and item 4 or 5. below, does not apply. ☐ The applicant has been informed accordingly.

4. ☐ The date of receipt of the demand is WITHIN the period of 19 months from the priority date as extended by virtue of Rule 80.5.

5. ☐ Although the date of receipt of the demand is after the expiration of 19 months from the priority date, the delay in arrival is EXCUSED pursuant to Rule 82.

For International Bureau use only

Demand received from IPEA on:

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

To: STEVEN B. KELBER
OBLON, SPIVAK, MCCLELLAND, MAIER & NEUSTADT
1755 JEFFERSON DAVIS HWY.
CRYSTAL SQUARE 5, 4TH FL.
ARLINGTON VA 22202

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

	Date of Mailing *(day/month/year)* 27 JAN 1998
Applicant's or agent's file reference 274/091/27CIP	FOR FURTHER ACTION **See paragraphs 1 and 4 below**
International application No. PCT/US97/15109	International filing date *(day/month/year)* 29 AUGUST 1997
Applicant CHANEY, RUFUS L.	

1. [X] The applicant is hereby notified that the international search report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the international search report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in rules 90 *bis* 1 and 90 *bis* 3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer M. ALEXANDRA ELVE
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0092

(See notes on accompanying sheet)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 274709127CIP	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
International application No. PCT/US97/15109	International filing date *(day/month/year)* 29 AUGUST 1997	(Earliest) Priority Date 30 AUGUST 1996
Applicant CHANEY, RUFUS L.		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 2 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. [ ] **Certain claims were found unsearchable** (See Box I).

2. [ ] **Unity of invention is lacking** (See Box II).

3. [ ] The international application contains disclosure of a **nucleotide and/or amino acid sequence listing and the** international search was carried out on the basis of the sequence listing

   [ ] filed with the international application.

   [ ] furnished by the applicant separately from the international application,

      [ ] but not accompanied by a statement to the effect that it did not include matter going beyond the disclosure in the international application as filed.

   [ ] transcribed by this Authority.

4. With regard to the title,

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is:

   Figure No. 2

   [ ] as suggested by the applicant.

   [X] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [ ] None of the figures.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/15109

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C22B 23/00
US CL :75/710
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 75/710, 392, 432; 210/602, 682, 688; 71/9

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,364,451 (RASKIN et al.) 15 November 1994, col. 1, lines 27-60.	1 & 11

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 02 DECEMBER 1997	Date of mailing of the international search report 27 JAN 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer M. ALEXANDRA ELVE Telephone No. (703) 308-0092

Form PCT/ISA/210 (second sheet)(July 1992)★

PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

International Application No.

International Filing Date

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference *(if desired) (12 characters maximum)* 274709127CIP

Box No. I TITLE OF INVENTION

METHOD FOR PHYTOMINING OF NICKEL, COBALT AND OTHER METALS FROM SOIL

Box No. II APPLICANT

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)*

CHANEY, Rufus L.
United States Department of Agriculture
Beltsville, Maryland 20705
US

☒ This person is also inventor.

Telephone No.

Facsimile No.

Teleprinter No.

State *(i.e. country)* of nationality: US

State *(i.e. country)* of residence: US

This person is applicant for the purposes of: ☒ all designated States ☐ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)*

ANGLE, Jay Scott
10241 Bristol Channel
Ellicot City, Maryland 21042
US

This person is:

☐ applicant only

☒ applicant and inventor

☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality: US

State *(i.e. country)* of residence: US

This person is applicant for the purposes of: ☒ all designated States ☐ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

☒ Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

KELBER, Steven B.
OBLON, SPIVAK, McCLELLAND, MAIER & NEUSTADT, P.C.
1755 Jefferson Davis Highway
Crystal Square Five, Fourth Floor
Arlington, Virginia 22202
US

Telephone No.
(703) 413-3000

Facsimile No.
(703) 413-2220

Teleprinter No.
248855 OPAT UR

☐ Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Continuation of Box No. III FURTHER APPLICANTS AND/OR (FURTHER) INVENTORS

If none of the following sub-boxes is used, this sheet is not to be included in the request.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)*

LI, Yin-Ming
12019 Coldstream Drive
Potomac, Maryland 20854
US

This person is:

☐ applicant only
☒ applicant and inventor
☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality: CN

State *(i.e. country)* of residence: US

This person is applicant for the purposes of: ☒ all designated States ☐ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)*

This person is:

☐ applicant only
☐ applicant and inventor
☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality:

State *(i.e. country)* of residence:

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)*

This person is:

☐ applicant only
☐ applicant and inventor
☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality:

State *(i.e. country)* of residence:

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)*

This person is:

☐ applicant only
☐ applicant and inventor
☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality:

State *(i.e. country)* of residence:

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

☐ Further applicants and/or (further) inventors are indicated on another continuation sheet.

Box No.V DESIGNATION OF STATES

The following designations are hereby made under Rule 4.9(a) *(mark the applicable check-boxes; at least one must be marked):*

Regional Patent

☒ AP **ARIPO Patent:** KE Kenya, LS Lesotho, MW Malawi, SD Sudan, SZ Swaziland, UG Uganda, and any other State which is a Contracting State of the Harare Protocol and of the PCT

☒ EA **Eurasian Patent:** AM Armenia, AZ Azerbaijan, BY Belarus, KG Kyrgyzstan, KZ Kazakstan, MD Republic of Moldova, RU Russian Federation, TJ Tajikistan, TM Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT

☒ EP **European Patent:** AT Austria, BE Belgium, CH and LI Switzerland and Liechtenstein, DE Germany, DK Denmark, ES Spain, FI Finland, FR France, GB United Kingdom, GR Greece, IE Ireland, IT Italy, LU Luxembourg, MC Monaco, NL Netherlands, PT Portugal, SE Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT

☒ OA **OAPI Patent:** BF Burkina Faso, BJ Benin, CF Central African Republic, CG Congo, CI Côte d'Ivoire, CM Cameroon, GA Gabon, GN Guinea, ML Mali, MR Mauritania, NE Niger, SN Senegal, TD Chad, TG Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT *(if other kind of protection or treatment desired, specify on dotted line)* .....

National Patent *(if other kind of protection or treatment desired, specify on dotted line):*

☒ AL Albania
☒ AM Armenia
☒ AT Austria
☒ AU Australia
☒ AZ Azerbaijan
☒ BA Bosnia and Herzegovina
☒ BB Barbados
☒ BG Bulgaria
☒ BR Brazil
☒ BY Belarus
☒ CA Canada
☒ CH and LI Switzerland and Liechtenstein
☒ CN China
☒ CU Cuba
☒ CZ Czech Republic
☒ DE Germany
☒ DK Denmark
☒ EE Estonia
☒ ES Spain
☒ FI Finland
☒ GB United Kingdom
☒ GE Georgia
☒ HU Hungary
☒ IL Israel
☒ IS Iceland
☒ JP Japan
☒ KE Kenya
☒ KG Kyrgyzstan
☒ KP Democratic People's Republic of Korea
☒ KR Republic of Korea
☒ KZ Kazakstan
☒ LC Saint Lucia
☒ LK Sri Lanka
☒ LR Liberia
☒ LS Lesotho
☒ LT Lithuania
☒ LU Luxembourg
☒ LV Latvia
☒ MD Republic of Moldova
☒ MG Madagascar
☒ MK The former Yugoslav Republic of Macedonia
☒ MN Mongolia
☒ MW Malawi
☒ MX Mexico
☒ NO Norway
☒ NZ New Zealand
☒ PL Poland
☒ PT Portugal
☒ RO Romania
☒ RU Russian Federation
☒ SD Sudan
☒ SE Sweden
☒ SG Singapore
☒ SI Slovenia
☒ SK Slovakia
☒ TJ Tajikistan
☒ TM Turkmenistan
☒ TR Turkey
☒ TT Trinidad and Tobago
☒ UA Ukraine
☒ UG Uganda
☒ US United States of America
☒ UZ Uzbekistan
☒ VN Viet Nam

Check-boxes reserved for designating States (for the purposes of a national patent) which have become party to the PCT after issuance of this sheet:

☒ GH Ghana
☒ SL Sierra Leone
☒ YU Yugoslavia
☒ ZW Zimbabwe

In addition to the designations made above, the applicant also makes under Rule 4.9(b) all designations which would be permitted under the PCT except the designation(s) of _____. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. *(Confirmation of a designation consists of the filing of a notice specifying that designation and the payment of the designation and confirmation fees. Confirmation must reach the receiving Office within the 15-month time limit.)*

Supplemental Box ***If the Supplemental Box is not used, this sheet need not be included in the request.***

Use this box in the following cases:

1. If, in any of the Boxes, the space is insufficient to furnish all the information:

in particular:

(i) if more than two persons are involved as applicants and/or inventors and no "continuation sheet" is available:

in such case, write "Continuation of Box No. III" and indicate for each additional person the same type of information as required in Box No. III;

(ii) if, in Box No. II or in any of the sub-boxes of Box No. III, the indication "the States indicated in the Supplemental Box" is checked:

in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the applicant(s) involved and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is applicant;

(iii) if, in Box No. II or in any of the sub-boxes of Box No. III, the inventor or the inventor/applicant is not inventor for the purposes of all designated States or for the purposes of the United States of America:

in such case write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the inventor(s) and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is inventor;

(iv) if, in addition to the agent(s) indicated in Box No. IV, there are further agents:

in such case, write "Continuation of Box No. IV" and indicate for each further agent the same type of information as required in Box No. IV;

(v) if, in Box No. V, the name of any State (or OAPI) is accompanied by the indication "patent of addition," or "certificate of addition," or if, in Box No. V, the name of the United States of America is accompanied by an indication "Continuation" or "Continuation-in-part":

in such case, write "Continuation of Box No. V" and the name of each State involved (or OAPI), and after the name of each such State (or OAPI), the number of the parent title or parent application and the date of grant of the parent title or filing of the parent application;

(vi) if there are more than three earlier applications whose priority is claimed:

in such case, write "Continuation of Box No. VI" and indicate for each additional earlier application the same type of information as required in Box No. VI.

2. If the applicant claims, in respect of any designated Office, the benefits of provisions of the national law concerning non-prejudicial disclosures or exceptions to lack of novelty:

in such case, write "Statement Concerning Non-Prejudicial Disclosures or Exceptions to Lack of Novelty" and furnish that statement below.

OBLON, Norman F.
SPIVAK, Marvin J.
McCLELLAND, C. Irvin
MAIER, Gregory J.
NEUSTADT, Arthur I.
KELLY, Richard D.
HAMILTON, James D.
KUESTERS, Eckhard H.
POUS, Robert T.
GHOLZ, Charles L.
SUNDERDICK, Vincent J.
BEAUMONT, William E.
GNUSE, Robert F.
LIPMAN, Steven E.
LAVALLEYE, Jean-Paul
BAXTER, Stephen G.
H A HL, Robert W.
TREANOR, Rrichard L.
ZOLTICK, Martin M.
WEIHROUCH, Steven P.
GOOLKASIAN, John T.
LABGOLD, Marc R.
HEALEY, William J.
CHINN, Richard L.
SCHLIER, Carl E.
KELBER, Steven B.
KULBASKI, James J.
RICHARDSON, Catherine B.
NEIFELD, Richard A.
MASON J. Derek

All of the Firm of:
OBLON, SPIVAK, McCLELLAND, MAIER & NEUSTADT, P.C.
Crystal Square Five, Fourth Floor
1755 Jefferson Davis Highway
Arlington, Virginia 22202, US

Box No. VI PRIORITY CLAIM Further priority claims are indicated in the Supplemental Box ☐

The priority of the following earlier application(s) is hereby claimed:

Country *(in which, or for which, the application was filed)*	Filing Date *(day/month/year)*	Application No.	Office of filing *(only for regional or international application)*
item (1) US	30 August 1996 (30-08-96)	60/024,928	
item (2) US	6 November 1996 (06-11-96)	60/030,462	
item (3)			

Mark the following check-box if the certified copy of the earlier application is to be issued by the Office which for the purposes of the present international application is the receiving Office (a fee may be required):

☒ The receiving Office is hereby requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) identified above as item(s): (1) and (2)

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(If two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used):* ISA/US

Earlier search *Fill in where a search (international, international-type or other) by the International Searching Authority has already been out or requested and the Authority is now requested to base the international search, to the extent possible, on the results of that earlier search, such search or request either by reference to the relevant application (or the translation thereof) or by reference to the search request:*

Country (or regional Office): Date *(day/month/year):* Number:

Box No. VIII CHECK LIST

This international application contains the following number of sheets:

1. request	:	5 sheets
2. description	:	35 sheets
3. claims	:	2 sheets
4. abstract	:	1 sheets
5. drawings	:	10 sheets
Total	:	53 sheets

This international application is accompanied by the item(s) marked below:

1. ☐ separate signed power of attorney
2. ☐ copy of general power of attorney
3. ☐ statement explaining lack of signature
4. ☐ priority document(s) *identified in Box No. VI as item(s):*
5. ☒ fee calculation sheet
6. ☐ separate indications concerning deposited microorganisms
7. ☐ nucleotide and/or amino acid sequence listing (diskette)
8. ☐ other *(specify):*

Figure No. _____ of the drawings (if any) should accompany the abstract when it is published.

Box No. IX SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).

William E. Beaumont

For receiving Office use only

1. Date of actual receipt of the purported international application:
2. Drawings ☐ received: ☐ not received:
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:
4. Date of timely receipt of the required corrections under PCT Article 11(2):
5. International Searching Authority specified by the applicant: ISA/
6. ☐ Transmittal of search copy delayed until search fee is paid

For International Bureau use only

Date of receipt of the record copy

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/15109

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C22B 23/00
US CL :75/710

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 75/710, 392, 432; 210/602, 682, 688; 71/9

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,364,451 (RASKIN et al.) 15 November 1994, col. 1, lines 27-60.	1 & 11

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 02 DECEMBER 1997	Date of mailing of the international search report 27 JAN 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer M. ALEXANDRA ELVE Telephone No. (703) 308-0092

Form PCT/ISA/210 (second sheet)(July 1992)

F ENT COOPERATION TREA

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

United States Patent and Trademark Office
(Box PCT)
Crystal Plaza 2
Washington, DC 20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day month year) 27 March 1998 (27.03.98)	
International application No. PCT/US97/15109	**Applicant's or agent's file reference** 274709127CIP
International filing date (day month year) 29 August 1997 (29.08.97)	**Priority date** (day month year) 30 August 1996 (30.08.96)
Applicant CHANEY, Rufus, L. et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

18 February 1998 (18.02.98)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer R. Raissi
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38